[18] T. Fukao, G-X Zhang, N. Sakura, T. Kubo, H. Yamaga, H. Hazama, Y. Kohno, N. Matsuo, M. Kondo, S. Yamaguchi, Y. Shigematsu, N. Kondo, The mitochondrial acetoacetyl-CoA thiolase deficiency in Japanese patients: urinary organic acid and blood acylcarnitine profiles under stable conditions have subtle abnormalities in T2-deficient patients with some residual T2 activity, J. Inherit. Metab. Dis. 26 (2003) 423–431.
[19] G.X. Zhang, T. Fukao, M.O. Rolland, M.T. Zabot, G. Renom, E. Touma, M. Kondo, N. Matsuo, N. Kondo, The mitochondrial acetoacetyl-CoA thiolase (T2) deficiency: T2-deficient patients with mild mutation(s) were previously misinterpreted as normal by the coupled assay with tiglyl-CoA, Pediatr. Res. 56 (2004) 60–64.
[20] L. Mrazova, T. Fukao, K. Halovd, E. Gregova, V. Kohut, D. Pribyl, P. Chrastina, N. Kondo, E. Pospisilova, Two novel mutations in mitochondrial acetoacetyl-CoA thiolase deficiency, J. Inherit. Metab. Dis. 28 (2005) 235–236.
[21] G.X. Zhang, T. Fukao, S. Sakurai, K. Yamada, K.M. Gibson, N. Kondo, Identification of Alu-mediated, large deletion-spanning exons 2–4 in a patient with mitochondrial acetoacetyl-CoA thiolase deficiency, Mol. Genet. Metab. 89 (2006) 222–226.
[22] S. Sakurai, T. Fukao, A.M. Haapalainen, G. Zhang, S. Yamada, F. Lilliu, S. Yano, P. Robinson, M.K. Gibson, R.J.A. Wanders, G.A. Mitchell, R.K. Wierenga, N. Kondo, Kinetic and expression analyses of seven novel mutations in mitochondrial acetoacetyl-CoA thiolase (T2): identification of a Km mutant and an analysis of the mutational sites in the structure, Mol. Genet. Metab. 90 (2007) 370–378.
[23] T. Fukao, G. Zhang, M-O. Rolland, M-T. Zabot, N. Guffon, Y. Aoki, N. Kondo, Identification of an Alu-mediated tandem duplication of exons 8 and 9 in a patient with mitochondrial acetoacetyl-CoA thiolase (T2) deficiency, Mol. Genet. Metab. 92 (2007) 375–378.
[24] T. Fukao, A. Boneh, Y. Aoki, N. Kondo, A novel single-base substitution (c.1124A>G) that activates a 5-base upstream cryptic splice donor site within exon 11 in the human mitochondrial acetoacetyl-CoA thiolase gene, Mol. Genet. Metab. 94 (2008) 417–421.
[25] S. Thümmler, D. Dupont, C. Acquaviva, T. Fukao, D. de Ricaud, Different clinical presentation in siblings with mitochondrial acetoacetyl-CoA thiolase deficiency and identification of two novel mutations, Tohoku J. Exp. Med. 220 (2010) 27–31.
[26] T. Fukao, X.Q. Song, S. Yamaguchi, T. Hashimoto, T. Orii, N. Kondo, Immunotitration analysis of cytocolic acetoacetyl-coenzyme A thiolase activity in human fibroblasts, Pediatr. Res. 39 (1996) 1055–1058.
[27] T. Fukao, X.Q. Song, G.A. Mitchell, S. Yamaguchi, K. Sukegawa, T. Orii, N. Kondo, Enzymes of ketone body utilization in human tissues: protein and mRNA levels of succinyl-CoA: 3-ketoacid CoA transferase and mitochondrial and cytosolic acetoacetyl-CoA thiolases, Pediatr. Res. 42 (1997) 498–502.
[28] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 192–200.
[29] H. Watanabe, K.E. Orii, T. Fukao, X-Q. Song, T. Aoyama, L. IJlst, J. Ruiter, R.J.A. Wanders, N. Kondo, Molecular basis of very long chain acyl-CoA dehydrogenase deficiency in three Israeli patients: identification of a complex mutant allele with P65L and K247Q mutations, the former being an exonic mutation causing exon 3 skipping, Hum. Mutat. 15 (2000) 430–438.
[30] P.J. Smith, C. Zhang, J. Wang, S.L. Chew, M.Q. Zhang, A.R. Krainer, An increased specificity score matrix for the prediction of SF2/ASF-specific exonic splicing enhancers, Hum. Mol. Genet. 15 (2006) 2490–2508.
[31] L. Cartegni, J. Wang, Z. Zhu, M.Q. Zhang, A.R. Krainer, ESEfinder: a web resource to identify exonic splicing enhancers, Nucleic Acid Res. 31 (2003) 3568–3571.
[32] A.C. Goldstrohm, A.L. Greenleaf, M.A. Garcia-Blanco, Co-transcriptional splicing of pre-messenger RNAs: considerations for the mechanism of alternative splicing, Gene 277 (2001) 31–47.
[33] T.A. Cooper, W. Mattox, The regulation of splice-site selection, and its role in human disease, Am. J. Hum. Genet. 61 (1997) 259–266.
[34] B.L. Robbertson, G.J. Cote, S.M. Berget, Exon definition may facilitate splice site selection in RNAs with multiple exons, Mol. Cell. Biol. 10 (1990) 84–94.
[35] M.B. Shapiro, P. Senapathy, RNA splice junctions of different classes of eukaryotes: sequence statistics and functional implications in gene expression, Nucleic Acids Res. 15 (1987) 7155–7174.
[36] S. Lin, X.D. Fu, SR proteins and related factors in alternative splicing, Adv. Exp. Med. Biol. 623 (2007) 107–122.
[37] A. Boichard, L. Venet, T. Naas, A. Boutron, L. Chevret, H.O. de Baulny, P. De Lonlay, A. Legrand, P. Nordman, M. Brivet, Two silent substitutions in the PDHA1 gene cause exon 5 skipping by disruption of a putative exonic splicing enhancer, Mol. Genet. Metab. 93 (2008) 323–330.
[38] V. Gonçalves, P. Theisen, O. Antunes, A. Medeira, J.S. Ramos, P. Jordan, G. Isidro, A missense mutation in the APC tumor suppressor gene disrupts an ASF/SF2 splicing enhancer motif and causes pathogenic skipping of exon 14, Mutat. Res. 662 (2009) 33–36.
[39] P.Y. Ho, M.Z. Huang, V.T. Fwu, S.C. Lin, K.J. Hsiao, T.S. Su, Simultaneous assessment of the effects of exonic mutations on RNA splicing and protein functions, Biochem. Biophys. Res. Commun. 373 (2008) 515–520.

J Inherit Metab Dis
DOI 10.1007/s10545-010-9168-5

RESEARCH REPORT

A neonatal-onset succinyl-CoA:3-ketoacid CoA transferase (SCOT)-deficient patient with T435N and c.658-666dupAACGTGATT p.N220_I222dup mutations in the *OXCT1* gene

Toshiyuki Fukao · Tomohiro Ishii · Naoko Amano · Petri Kursula · Masaki Takayanagi · Keiko Murase · Naomi Sakaguchi · Naomi Kondo · Tomonobu Hasegawa

Received: 30 March 2010 / Revised: 30 June 2010 / Accepted: 2 July 2010

Abstract Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency causes episodic ketoacidotic crises and no apparent symptoms between them. Here, we report a Japanese case of neonatal-onset SCOT deficiency. The male patient presented a severe ketoacidotic crisis, with blood pH of 7.072 and bicarbonate of 5.8 mmol/L at the age of 2 days and was successfully treated with intravenous infusion of glucose and sodium bicarbonate. He was diagnosed as SCOT deficient by enzymatic assay and mutation analysis. At the age of 7 months, he developed a second ketoacidotic crisis, with blood pH of 7.059, bicarbonate of 5.4 mmol/L, and total ketone bodies of 29.1 mmol/L. He experienced two milder ketoacidotic crises at the ages of 1 year and 7 months and 3 years and 7 months. His urinary ketone bodies usually range from negative to 1+ but sometimes show 3+ (ketostix) without any symptoms. Hence, this patient does not show permanent ketonuria, which is characteristic of typical SCOT-deficient patients. He is a compound heterozygote of c.1304C > A (T435N) and c.658-666dupAACGTGATT p.N220_I222dup. mutations in the *OXCT1* gene. The T435N mutation was previously reported as one which retained significant residual activity. The latter novel mutation was revealed to retain no residual activity by

Communicated by: K. Michael Gibson

References to electronic databases: Succinyl-CoA:3-ketoacid CoA transferase deficiency (OMIM 245050 601424): Succinyl-CoA:3-ketoacid CoA transferase (EC 2.8.3.5): *OXCT1* gene (gene ID 5019 NM_000436)

Competing interests: None declared.

T. Fukao · K. Murase · N. Sakaguchi · N. Kondo
Department of Pediatrics, Graduate School of Medicine,
Gifu University,
1-1 Yanagido,
Gifu, Gifu 501-1194, Japan

T. Fukao (✉)
Medical Information Sciences Division,
United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University,
1-1 Yanagido,
Gifu, Gifu 501-1194, Japan
e-mail: toshi-gif@umin.net

T. Fukao
Clinical Research Division, Nagara Medical Center,
Gifu, Gifu 502-8558, Japan

T. Ishii · N. Amano · T. Hasegawa
Department of Pediatrics, Keio University School of Medicine,
Shinjuku, Tokyo 160-8582, Japan

P. Kursula
Department of Biochemistry, University of Oulu,
P.O. Box 3000, FIN-90014 Oulu, Finland

M. Takayanagi
Chiba Children's Hospital,
Chiba, Chiba 266-0007, Japan

Published online: 21 July 2010

transient expression analysis. Both T435N and N220_I222 lie close to the SCOT dimerization interface and are not directly connected to the active site in the tertiary structure of a human SCOT dimer. In transient expression analysis, no apparent interallelic complementation or dominant negative effects were observed. Significant residual activity from the T435N mutant allele may prevent the patient from developing permanent ketonuria.

Abbreviations

SCOT succinyl-CoA:3-ketoacid CoA transferase
TKB total ketone bodies
FFA free fatty acids

Introduction

Ketone bodies, produced mainly in the liver, are an important source of energy for extrahepatic tissues (Mitchell and Fukao 2001). Succinyl-CoA: 3-ketoacid CoA transferase (SCOT; EC 2.8.3.5) is a mitochondrial homodimer essential for ketone body utilization. SCOT deficiency (OMIM 245050) causes episodic ketoacidosis and is part of the differential diagnosis of childhood ketoacidosis, a frequently occurring condition. In contrast with most organic acidemias, no diagnostic metabolites are observed in the blood and urine samples from SCOT-deficient patients, although the ketone bodies, acetoacetate, and 3-hydroxybutyrate are elevated (Mitchell and Fukao 2001). Since the first description of SCOT deficiency (Cornblath et al. 1971; Tildon and Cornblath 1972), fewer than 30 affected probands have been reported, including personal communication (Saudubray et al. 1987; Perez-Cerda et al. 1992; Sakazaki et al. 1995; Fukao et al. 1996, 2000, 2004, 2006; Pretorius et al. 1996; Niezen-Koning et al. 1997; Rolland et al. 1998; Snyderman et al. 1998; Berry et al. 2001; Longo et al. 2004; Yamada et al. 2007; Merron and Akhtar 2009; and seven other unpublished cases sent to TF). Because of the nonspecific metabolite profile of SCOT-deficient patients, in vitro methods of diagnosis are particularly important. Enzyme assays of SCOT activity are sufficient for clinical diagnosis, but current whole-cell assays can yield a spuriously high apparent residual activity (Perez-Cerda et al. 1992; Sakazaki et al. 1995; Kassovska-Bratinova et al. 1996). To assist clinical diagnosis, we cloned the human SCOT complementary DNA (cDNA) (Kassovska-Bratinova et al. 1996) and SCOT gene (*OXCT1*, Fukao et al. 2000), developed an anti-(human SCOT) antibody (Song et al. 1997), and described ten *OXCT1* gene mutations in SCOT-deficient patients (Kassovska-Bratinova et al. 1996; Song et al. 1998; Fukao et al. 2000, 2004, 2006, 2007; Longo et al. 2004; Yamada et al. 2007).

SCOT deficiency is one of the most important differential diagnosis of neonatal ketoacidotic crisis, since about half of the reported SCOT-deficient patients developed their first ketoacidotic crises in the neonatal period (Mitchell and Fukao 2001). Persistent ketosis and ketonuria are pathognomonic features of SCOT deficiency; however, these are not present in all SCOT-deficient patients. We previously pointed out that patients with mutation T435N, which retained some residual SCOT activity, do not show permanent ketosis (Fukao et al. 2004). In this study, we describe a Japanese SCOT-deficient patient with neonatal onset. One of his mutations was revealed to be T435N.

Materials and methods

Case presentation

The proband (GS21) is a Japanese boy born from non-consanguineous parents at 38 weeks of gestation. The pregnancy and delivery were uneventful. His birth length was 49.1 cm (50th–90th percentile), weight 2.59 kg (3rd–10th percentile), and head circumference 34.3 cm (50th–90th percentile). At the age of 2 days, he presented tachypnea and poor drinking ability. Physical examination revealed grunting and sternal retraction with a respiration rate of 60/min. Blood gas analysis showed severe metabolic acidosis [pH 7.072, partial pressure of carbon dioxide (PCO_2) 20.5 mmHg, bicarbonate 5.8 mmol/L]. The blood glucose level was 3 mmol/L, ammonia 95 μmol/L, sodium (Na) 151 mEq/L, potassium (K) 4.19 mEq/L, chlorine (Cl) 113.1 mEq/L, and urinary ketone bodies 3+ (ketostix, Siemens Healthcare Diagnostics, USA). GS21 was treated by intravenous infusion of glucose and sodium bicarbonate. Urinary organic-acid analysis by gas chromatography-mass spectrometry showed massive amounts of 3-hydroxybutyrate and acetoacetate with dicarboxylic acids. He was transferred to Keio University Hospital for further evaluation at 4 days of age. On admission, his general condition and blood gas data (pH 7.453, PCO_2 24.4 mmHg, bicarbonate 20.4 mmol/L) improved with intravenous infusion of glucose at 7 mg/kg/min. GS21 was suspected of having SCOT deficiency, and this was confirmed by enzyme assay using peripheral blood mononuclear cells and mutation analysis (see "Results and Discussion"). At the age of 3 weeks, serum free fatty acids (FFA) and total ketone bodies (TKB) were measured at 1, 3, and 6 h after feeding, as shown in Table 1. At the age of 1 month, the boy was discharged from the hospital. The feeding interval was kept at <6 h to avoid severe ketoacidosis.

At the ages of 3 months and 4 months, FFA and TKB (3 h after feeding) were measured, as shown in Table 1. TKB levels became higher than those at the age of 3 weeks.

Table 1 Serum free fatty acids (FFA) and total keytone bodies (TKB)

Age		FFA (mM)	TKB (mM)	FFA/TKB
3 weeks	1 h after feeding	0.20	0.24	0.83
	3 h after feeding	0.17	0.15	1.13
	6 h after feeding	0.46	0.81	0.57
3 months	3 h after feeding	0.53	2.54	0.21
4 months	3 h after feeding	0.39	1.49	0.26
7 months	Ketoacidotic crisis	1.84	29.1	0.06
	2 days after crisis	0.97	0.54	1.80
1.5 years	Mild ketoacidotic crisis	ND	10.3	NA
3 years 7 months	Mild ketoacidotic crisis	1.61	11.2	0.14
Reference values*	Fed state		0.10-0.30	
	15-h fast	0.5-1.6	0.10-0.70	0.6-5.2

ND not determined, *NA* not applicable

*Reference values are from Bonnefont et al. 1990

At the age of 7 months, he developed his second ketoacidotic crisis for no clear reason and was again hospitalized. The laboratory findings were blood pH 7.059, PCO_2 20.2 mmHg, bicarbonate 5.4 mmol/L, glucose 2.2 mmol/L, and TKB 29.1 mmol/L. Treatment was begun with intravenous infusion of glucose at 5.5–7.1 mg/kg/min. He had a bolus injection of 1 mEq/kg of sodium bicarbonate followed by continuous infusion of sodium bicarbonate at 1 mEq/kg/h for 8 h. Thirteen hours after admission, continuous infusion of insulin was also initiated at a glucose/insulin ratio of 6.2–8.3 g/U, since his blood glucose levels were rather high (11.0 mmol/L). The urinary ketone bodies turned negative 2 days after admission. At the ages of 1 year 7 months and 3 years 7 months, he exhibited his third and fourth episodes of ketoacidotic crisis due to acute gastroenteritis. Blood pH was 7.280, PCO_2 20.3 mmHg, bicarbonate 9.2 mmol/L, and TKB 10.3 mmol/L in the third episode, and blood pH was 7.192, PCO_2 17.3 mmHg, bicarbonate 6.4 mmol/L in the fourth episode. FFA and TKB during the episodes are shown in Table 1. GS21 recovered by continuous infusion of glucose only during both episodes. At the age of 3 years and 5 months, he was 93.4 cm in height (50th percentile), 11.95 kg in weight (10th percentile), and had a head circumference of 50.4 cm (50th–75th percentile). The patient is now 3 years and 9 months old, and his motor and mental development are within normal range. The feeding interval has been prolonged up to 12 h. His urinary ketone bodies usually range from negative to 1+ but sometimes show 3+ without any symptoms.

Enzyme assay

Informed consent for enzymatic diagnosis and molecular analysis was obtained from the parents of GS21. This study was approved by the Ethical Committee of the Graduate School of Medicine, Gifu University. Assays for acetoacetyl-CoA thiolase and for SCOT were as previously described (Fukao et al. 1997; Song et al. 1997) using acetoacetyl-CoA as a substrate and measuring its disappearance spectrophotometrically.

Mutation analysis

Total RNA was purified from peripheral blood mononuclear cells with an ISOGEN kit (Nippon Gene, Tokyo, Japan). Real-time polymerase chain reaction (RT-PCR) was as previously described (Kassovska-Bratinova et al. 1996). Mutations were detected by amplifying cDNA spanning the full-length coding sequence and by sequencing ten clones. Genomic DNA was purified with a Sepa Gene kit (Sanko Junyaku, Tokyo, Japan). Mutation analysis at the genomic level was done by PCR for each exon and its intron boundaries (at least 50 bases from the exon/intron boundaries for both directions), followed by direct sequencing (Fukao et al. 2000).

Transient expression analysis

Mutant expression vectors were made using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA) and were confirmed by sequencing. Wild-type and mutant SCOT expression vectors (4 μg) were first transfected using Lipofectamine 2000 (GIBCO BRL Invitrogen Inc., Carlsbad, CA, USA) in ~10^5 SV40-transformed SCOT-deficient fibroblasts of GS01 (Kassovska-Bratinova et al. 1996; Fukao et al. 2004). One microgram of the cytosolic acetoacetyl-CoA thiolase (CT)-expressing vector, pCAGGSct (Song et al. 1994), was cotransfected to monitor transfection efficiency. Transfection was done at 37°C for 24 h, then a further 48-h incubation was done at 37°C. The cells were harvested and stored at –80°C until SCOT and CT activities were assayed. Immunoblotting was done using a mixture of an anti-(human SCOT) antibody

(Song et al. 1997) and anti-(human CT) antibody (Song et al. 1994) as the first antibody (Fukao et al. 1997). The quantity of the mutant protein was estimated densitometrically, comparing it to the signal intensities of serially diluted samples of the wild-type SCOT protein.

Structural analysis

For analyzing putative structural effects of mutations on the SCOT protein, the recently determined crystal structure from the Structural Genomics Consortium [Protein Data Bank (PDB) entry 3DLX] of human SCOT was taken as a starting point. The structure was subjected to further crystallographic refinement using PHENIX (Adams et al. 2010) and COOT (Emsley and Cowtan 2004), including the addition of missing side chains and rebuilding the solvent network.

Results and discussion

Molecular diagnosis and characterization of mutations

SCOT activity in GS21's peripheral blood mononuclear cells was apparently much lower than two controls (GS21:0.25, control 1: 2.4, control 2: 3.9 nmol/min/mg of protein). Hence, we tentatively diagnosed him as having SCOT deficiency. Since we could not draw blood for a repeat of the enzyme assay at the age of 1 month and skin biopsy was not acceptable to the parents, we performed mutation analysis to confirm the diagnosis. The full coding sequence of SCOT cDNA from GS21 was sequenced after subcloning. Mutations c.658-666dupAACGTGATT p.N220_I222dup. and c.1304C > A (T435N) were separately identified in six and four clones, respectively. No other mutations were found at the cDNA level. We

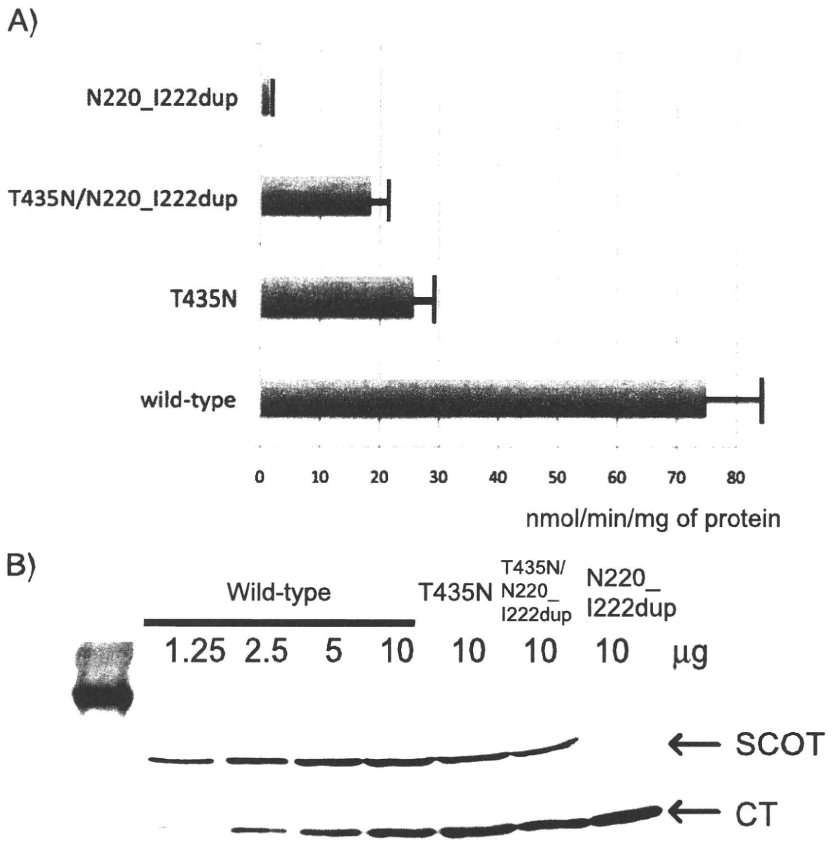

Fig. 1 Transient expression analysis of T435N and N220_I222dup mutant complementary DNAs (cDNAs). Transient expression analysis was performed at 37°C. Expression vectors (4 μg) were transfected. *T435N/ N220_I222dup* indicates a cotransfection of 4 μg each of two mutant expression vectors for T435N and N220_I222dup. **a** Succinyl-CoA:3-ketoacid CoA transferase (SCOT) enzyme assay. SCOT activity in the supernatant of the cell extract was measured. The mean values are shown together with the standard deviation (SD) of three independent experiments. **b** Immunoblot analysis. The protein amounts applied are shown *above the lanes*. We used previously described rabbit polyclonal antibodies, which we made (Song et al. 1994, 1997), and ProtoBlot Western blot AP system (Promega, Madison, WI, USA). The first antibody was a mixture of an anti-human cytosolic thiolase (CT) antibody and anti-human SCOT antibody. The positions of the bands for CT and SCOT are indicated by *arrows*. Immunoblotting was done using a mixture of an anti-(human SCOT) antibody (Song et al. 1997) and anti-(human CT) antibody (Song et al. 1994) as the first antibody (Fukao et al. 1997)

confirmed these mutations at the genomic level. Familial analysis showed that the 9-bp duplication was inherited from the mother and c.1304C > A (T435N) from the father. The 9-bp duplication was a novel mutation and T435N was previously reported in Japanese patients (Fukao et al. 2004).

Transient expression analysis of the c.658-666dupAACGT GATT p.N220_I222dup cDNA showed no residual activity (Fig. 1a), and the N220_I222dup SCOT protein was not detected in immunoblot analysis (Fig. 1b). Since SCOT protein is a homodimer and the T435N mutant retains significant residual SCOT activity, as previously reported (Fukao et al. 2004), we investigated possible interallelic influence between T435N and N220_I222dup by cotransfection of these two mutant cDNAs. However, no apparent interallelic complementation or dominant negative effect were observed.

The T435N mutation was previously identified in two Japanese SCOT-deficient families from the Amami islands (Fukao et al. 2004). There was no apparent consanguinity or relationship between these two families, and the patients were homozygotes of T435N. Since the Amami islands have a population of about 120,000, this mutation might be prevalent in that region. However, the father of GS21, who carries the T435N mutation, has no relation to the islands as far as he knows.

Recently, the crystal structure of human SCOT was determined (PDB entry 3DLX). The SCOT protein is a homodimer; both T435 and N220_I222 lie close to the SCOT dimerization interface and are not directly connected to the active site. Their overall localization in the context of the dimer is shown in Fig. 2a. T435 lies in a loop, with the side chain pointing inward to a rather hydrophilic environment (Fig. 2b); the hydroxyl group makes a hydrogen bond with a backbone carbonyl group from V394. Thus, it is expected that a T435N substitution will perturb the SCOT structure only a little, being able to make hydrogen bond interactions. This is also reflected in the fact that the T435N mutation is associated with significant residual SCOT activity. NVI220-222 is a short beta strand from a beta sandwich domain involved in SCOT dimerization (Fig. 2c). This small domain also contains other previously identified SCOT point mutations, namely, G219E, V221M, R224K, and R268H (Fukao et al. 2000, 2007; Yamada et al. 2007), making it a hot-spot for SCOT mutations. The small beta sandwich is tightly folded, and

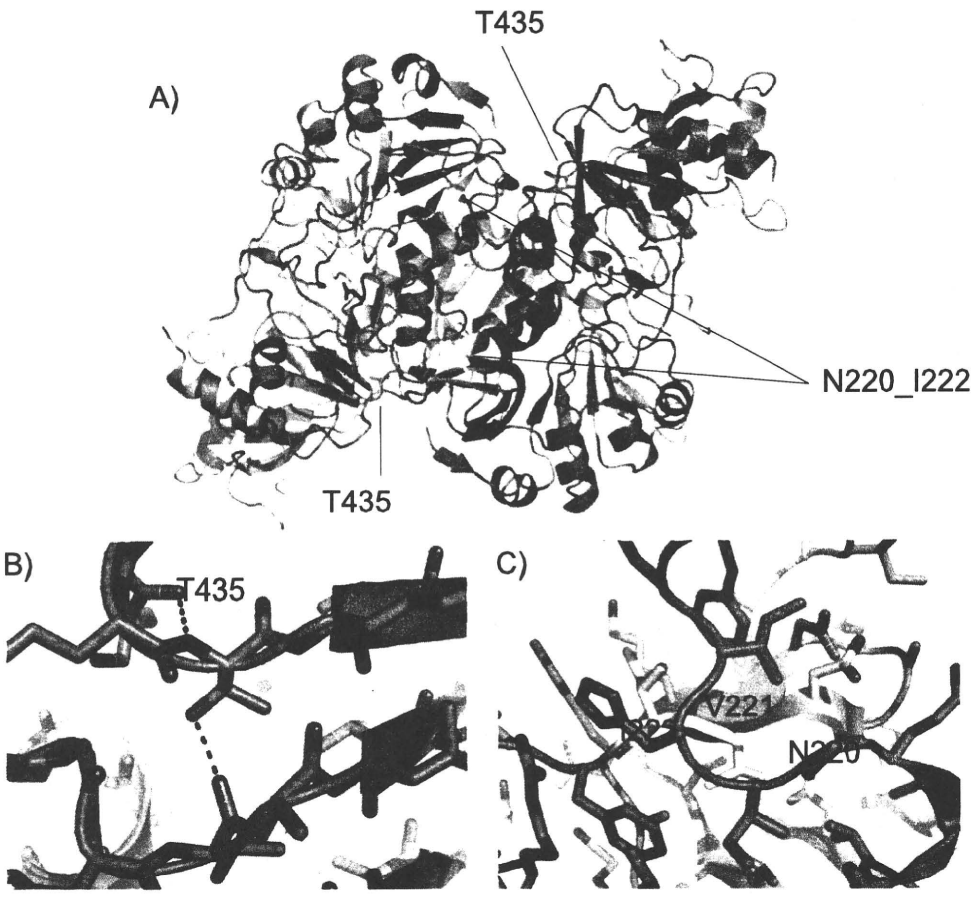

Fig. 2 Tertiary structure around mutations. **a** A human succinyl-CoA:3-ketoacid CoA transferase (SCOT) dimer, with E344 (highlighting the active site of SCOT) in *blue*, N220_I222 in *yellow*, and T435 in *orange*. The two monomers are shown in *green and light blue*. **b** Detailed surroundings of T435 (*orange*). Note the side chain is buried in a hydrophilic environment, making a hydrogen bond with a backbone oxygen. There are also additional potential hydrogen bonding partners in the vicinity. **c** The N220_I222 (in *yellow*) correspond to a short buried beta strand in a tightly packed environment. The dimer interface is at the *top* in this view

one can expect a duplication of three residues within the beta strand to disrupt its folding. This might affect the overall folding of the SCOT monomer and/or the formation of functional SCOT dimers.

Clinical issues

GS21 developed his first ketoacidotic crisis at the age of 2 days, although one of his mutated alleles retained residual SCOT activity. Almost half of the patients with SCOT deficiency develop their first ketoacidotic crisis at the age of 2–4 days (Mitchell and Fukao 2001). We summarized the mutations and their clinical phenotypes for several SCOT-deficient patients, including GS21, in Table 2. GS10, a homozygote of the R268H mutation, which retained residual activity, also developed his first ketoacidotic crisis at the age of 2 days, whereas his sibling (GS10s) with the same mutation developed her first crisis at the age of 6 months (Fukao et al. 2007). On the other hand, GK15, a homozygote of the null mutation, R217X, developed her first ketoacidotic crisis at the age of 8 months (Longo et al. 2004). Neonatal onset, hence, does not appear to be related to residual SCOT activity.

Permanent ketosis, or ketonuria, is a pathognomonic feature of SCOT deficiency (Mitchell and Fukao 2001). We previously reported that patients (GS08, GS09, and GS09b) who are homozygous for T435N did not show permanent ketosis or ketonuria (Fukao et al. 2004). In the case of GS21, the blood levels of FFA and TKB at 3 h after feeding were measured at the ages of 3 weeks, 3 monthsm and 4 months (Table 1). The level of TKB at the age of 3 weeks (0.15 mmol/L) was much less than those at the ages of 3 months and 4 months (2.54, 1.59 mmol/L, respectively). The FFA/TKB ratio at the ages of 3 and 4 months was 0.21 and 0.26, respectively, but was nearly 1.0 at the age of 3 weeks. In the cases of SCOT-deficient patients, this ratio was reported to be <0.3 early in a fasting test (Bonnefont et al. 1990). Even at 6 h after feeding at the age of 3 weeks, the level of TKB was 0.81 and the ratio was 0.57. These facts may mean that a hyperketotic status is not apparent during a nonepisodic condition in the neonatal period. The blood levels of FFA and TKB were not available during the age of 2–3 years, but urinary ketone bodies varied from negative to 3+ without any symptoms in GS21 and were always positive in GS02 and GS02s, whose mutations do not retain residual SCOT activity (Sakazaki et al. 1995). Hence, GS21 has no permanent ketonuria. It is very important to state that SCOT deficiency is the most probable diagnosis if permanent ketosis/ketonuria is present but that SCOT deficiency is not excluded even if permanent ketosis/ketonuria is absent.

Table 2 Succinyl-CoA:3-ketoacid CoA transferase (SCOT)-deficient patients

Number	Country	Sex	Mutations	Res. activity[a]	Onset	First ketoacidotic crisis				PK[b]	No. of episodes	Prognosis	References	
						pH	HCO3	TKB	glucose					
GS02	Japan	M	V133E/C456F	–	6mo	7.08	5.1	12.2	6.7	+	3	Good	Sakazaki et al. 1995	Song et al. 1998
GS02s	Japan	F	V133E/C456F	–	prenatal diagn.					+	1	Good	Fukao et al. 1996	Song et al. 1998
GS10	South Africa	M	R268H/R268H	+	2d	6.94	5	14.2	4.4	–	>5	Good	Pretorius et al. 1996	Fukao et al. 2007
GS10s	South Africa	F	R268H/R268H	+	6mo	7.12	8	5.1	6.6	–	5	Good	Pretorius et al. 1996	Fukao et al. 2007
GS15	USA	F	R217X/R217X	–	8mo	6.98	<5			+	1	Good	Longo et al. 2004	
GS08	Japan	M	T435N/T435N	+	1y5m	7.12	3.7	18.5	6.0	–	3	Good	Fukao et al. 2004	
GS09	Japan	M	T435N/T435N	+	10mo	7.00	5.8			–	>5	Good	Fukao et al. 2004	
GS09b	Japan	M	T435N/T435N	+	10mo	7.09	5.4		7.1	–	4	Good	Fukao et al. 2004	
GS21	Japan	M	T435N/N220_I222dup	+	2d	7.02	5.8		3	–	3	Good	This study	

[a] Residual SCOT activity in transient expression analysis of mutant complementary DNAs, [b] permanent ketosis

Acknowledgements This study was in part supported by Health and Labor Science Research Grants for Research on Intractable Diseases and Research on Children and Families from The Ministry of Health, Labor and Welfare of Japan and by á Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan

References

Adams PD, Afonine PV, Bunkoczi G et al (2010) PHENIX: a comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr D Biol Crystallogr 66:213–221

Berry GT, Fukao T, Mitchell GA et al (2001) Neonatal hypoglycaemia in severe succinyl-CoA: 3-oxoacid CoA-transferase deficiency. J Inherit Metab Dis 24:587–595

Bonnefont JP, Specola NB, Vassault A et al (1990) The fasting test in paediatrics: application to the diagnosis of pathological hypo- and hyperketotic states. Eur J Pediatr 150:80–85

Cornblath M, Gingell RL, Fleming GA, Tildon JT, Leffler AT, Wapnir RA (1971) A new syndrome of ketoacidosis in infancy. J Pediatr 79:413–418

Emsley P, Cowtan K (2004) Coot: model-building tools for molecular graphics. Acta Crystallogr D Biol Crystallogr 60:2126–2132

Fukao T, Song XQ, Watanabe H et al (1996) Prenatal diagnosis of succinyl-coenzyme A:3-ketoacid coenzyme A transferase deficiency. Prenat Diagn 16:471–474

Fukao T, Song XQ, Mitchell GA et al (1997) Enzymes of ketone body utilization in human tissues: protein and messenger RNA levels of succinyl-coenzyme A (CoA):3-ketoacid CoA transferase and mitochondrial and cytosolic acetoacetyl-CoA thiolases. Pediatr Res 42:498–502

Fukao T, Mitchell GA, Song XQ et al (2000) Succinyl-CoA:3-ketoacid CoA transferase (SCOT): cloning of the human SCOT gene, tertiary structural modeling of the human SCOT monomer, and characterization of three pathogenic mutations. Genomics 68:144–151

Fukao T, Shintaku H, Kusubae R et al (2004) Patients homozygous for the T435N mutation of succinyl-CoA:3-ketoacid CoA Transferase (SCOT) do not show permanent ketosis. Pediatr Res 56:858–863

Fukao T, Sakurai S, Rolland MO et al (2006) A 6-bp deletion at the splice donor site of the first intron resulted in aberrant splicing using a cryptic splice site within exon 1 in a patient with succinyl-CoA: 3-Ketoacid CoA transferase (SCOT) deficiency. Mol Genet Metab 89:280–282

Fukao T, Kursula P, Owen EP, Kondo N (2007) Identification and characterization of a temperature-sensitive R268H mutation in the human succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene. Mol Genet Metab 92:216–221

Kassovska-Bratinova S, Fukao T, Song XQ et al (1996) Succinyl CoA: 3-oxoacid CoA transferase (SCOT): human cDNA cloning, human chromosomal mapping to 5p13, and mutation detection in a SCOT-deficient patient. Am J Hum Genet 59:519–528

Longo N, Fukao T, Singh R et al (2004) Succinyl-CoA:3-ketoacid transferase (SCOT) deficiency in a new patient homozygous for an R217X mutation. J Inherit Metab Dis 27:691–692

Merron S, Akhtar R (2009) Management and communication problems in a patient with succinyl-CoA transferase deficiency in pregnancy and labour. Int J Obstet Anesth 18:280–283

Mitchell GA, Fukao T (2001) Chapter 102. Inborn errors of ketone body catabolism. In: Scriver CR, Beaudet AL, Sly WS, Valle D (eds) Metabolic and molecular bases of inherited disease, 8th edn. McGraw-Hill Inc, New York, pp 2327–2356

Niezen-Koning KE, Wanders RJ, Ruiter JP et al (1997) Succinyl-CoA: acetoacetate transferase deficiency: identification of a new patient with a neonatal onset and review of the literature. Eur J Pediatr 156:870–873

Perez-Cerda C, Merinero B, Sanz P et al (1992) A new case of succinyl-CoA: acetoacetate transferase deficiency. J Inherit Metab Dis 15:371–373

Pretorius CJ, Loy Son GG, Bonnici F, Harley EH (1996) Two siblings with episodic ketoacidosis and decreased activity of succinyl-CoA:3-ketoacid CoA-transferase in cultured fibroblasts. J Inherit Metab Dis 19:296–300

Rolland MO, Guffon N, Mandon G, Divry P (1998) Succinyl-CoA: acetoacetate transferase deficiency. Identification of a new case; prenatal exclusion in three further pregnancies. J Inherit Metab Dis 21:687–688

Sakazaki H, Hirayama K, Murakami S et al (1995) A new Japanese case of succinyl-CoA: 3-ketoacid CoA-transferase deficiency. J Inherit Metab Dis 18:323–325

Saudubray JM, Specola N, Middleton B, Lombes A, Bonnefont JP, Jakobs C, Vassault A, Charpentier C, Day R (1987) Hyperketotic states due to inherited defects of ketolysis. Enzyme 38:80–90

Snyderman SE, Sansaricq C, Middleton B (1998) Succinyl-CoA:3-ketoacid CoA-transferase deficiency. Pediatrics 101:709–711

Song XQ, Fukao T, Yamaguchi S, Miyazawa S, Hashimoto T, Orii T (1994) Molecular cloning and nucleotide sequence of complementary DNA for human hepatic cytosolic acetoacetyl-coenzyme A thiolase. Biochem Biophys Res Commun 201:478–485

Song XQ, Fukao T, Mitchell GA et al (1997) Succinyl-CoA:3-ketoacid coenzyme A transferase (SCOT): development of an antibody to human SCOT and diagnostic use in hereditary SCOT deficiency. Biochim Biophys Acta 1360:151–156

Song XQ, Fukao T, Watanabe H et al (1998) Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency: two pathogenic mutations, V133E and C456F, in Japanese siblings. Hum Mutat 12:83–88

Tildon JT, Cornblath M (1972) Succinyl-CoA: 3-ketoacid CoA-transferase deficiency. A cause for ketoacidosis in infancy. J Clin Invest 51:493–498

Yamada K, Fukao T, Zhang G et al (2007) Single-base substitution at the last nucleotide of exon 6 (c.671G > A), resulting in the skipping of exon 6, and exons 6 and 7 in human succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene. Mol Genet Metab 90:291–297

Tohoku J. Exp. Med., 2010, **221**, 191-195

Carnitine Palmitoyltransferase 2 Deficiency: The Time-Course of Blood and Urinary Acylcarnitine Levels during Initial L-Carnitine Supplementation

Tomohiro Hori,[1] Toshiyuki Fukao,[1,2,3] Hironori Kobayashi,[4] Takahide Teramoto,[1] Masaki Takayanagi,[5] Yuki Hasegawa,[4] Tetsuhiko Yasuno,[6] Seiji Yamaguchi[4] and Naomi Kondo[1]

[1]Department of Pediatrics, Gifu University Graduate School of Medicine, Gifu, Japan
[2]Medical Information Science Division, United Graduate School of Drug Discovery and Medical Information, Gifu University, Gifu, Japan
[3]Clinical Research Division, Nagara Medical Center, Gifu, Japan
[4]Department of Pediatrics, Shimane University School of Medicine, Shimane, Japan
[5]Department of Metabolism, Chiba Children's Hospital, Chiba, Japan
[6]Division of Nephrology and Rheumatology, Department of Internal Medicine, Fukuoka University School of Medicine, Fukuoka, Japan

Carnitine palmitoyltransferase 2 (CPT2) deficiency is one of the most common mitochondrial beta-oxidation defects. A female patient with an infantile form of CPT2 deficiency first presented as having a Reye-like syndrome with hypoglycemic convulsions. Oral L-carnitine supplementation was administered since serum free carnitine level was very low (less than 10 μmol/L), indicating secondary carnitine deficiency. Her serum and urinary acylcarnitine profiles were analyzed successively to evaluate time-course effects of L-carnitine supplementation. After the first two days of L-carnitine supplementation, the serum level of free carnitine was elevated; however, the serum levels of acylcarnitines and the urinary excretion of both free carnitine and acylcarnitines remained low. A peak of the serum free carnitine level was detected on day 5, followed by a peak of acetylcarnitine on day 7, and peaks of long-chain acylcarnitines, such as C16, C18, C18:1 and C18:2 carnitines, on day 9. Thereafter free carnitine became predominant again. These peaks of the serum levels corresponded to urinary excretion peaks of free carnitine, acetylcarnitine, and medium-chain dicarboxylic carnitines, respectively. It took several days for oral L-carnitine administration to increase the serum carnitine levels, probably because the intracellular stores were depleted. Thereafter, the administration increased the excretion of abnormal acylcarnitines, some of which had accumulated within the tissues. The excretion of medium-chain dicarboxylic carnitines dramatically decreased on day 13, suggesting improvement of tissue acylcarnitine accumulation. These time-course changes in blood and urinary acylcarnitine levels after L-carnitine supplementation support the effectiveness of L-carnitine supplementation to CPT2-deficient patients.

Keywords: carnitine palmitoyltransferase 2; CPT2; L-carnitine; acylcarnitine profile; carnitine administration
Tohoku J. Exp. Med., 2010, **221** (3), 191-195.

Carnitine palmitoyltransferase 2 (CPT2) deficiency (EC 2.3.1.21, OMIM 600650) is one of the most common disorders of mitochondrial fatty acid oxidation. CPT2 deficiency has several clinical presentations (Bonnefont et al. 1999). The adult form is characterized by episodes of rhabdomyolysis triggered by prolonged exercise. The infantile form presents as severe attacks of hypoketotic hypoglycemia, occasionally associated with sudden infant death or a Reye-like syndrome (Demaugre et al. 1991; Hug et al. 1991). The most severe kind, the neonatal form, is almost always lethal during the first month of life.

Secondary carnitine deficiency, characterized by low levels of total and free carnitines associated with an increase in the long-chain acylcarnitine fraction, is observed in the infantile form of CPT2-deficient patients (Bonnefont et al. 2004; Longo et al. 2006). Hence, L-carnitine supply might be useful in severe CPT2 deficiencies (Bonnefont et al. 2004), although supplementation with L-carnitine in patients with beta-oxidation defects of long-chain acyl-CoA has long been a matter of controversy (Costa et al. 1998;

Received March 23, 2010; revision accepted for publication May 17, 2010. doi:10.1620/tjem.221.191
Correspondence: Toshiyuki Fukao, M.D., Ph.D., Department of Pediatrics, Gifu University Graduate School of Medicine, Yanagido 1-1, Gifu 501-1194, Japan.
e-mail:toshi-gif@umin.net

Liebig et al. 2006; Primassin et al. 2008).

In this report, we describe a CPT2-deficient patient who presented as having a Reye-like syndrome with secondary carnitine deficiency. We focused on time-dependent changes in the serum and urinary acylcarnitine profiles after intial L-carnitine supplementation.

Clinical Report

The patient, a female, was born to nonconsanguineous Japanese parents. She had been well until 15 months of age when she suddenly had tonic-clonic convulsions at 3:00 a.m. for about 30 minutes and became unconscious. Ten days before the convulsions, she had a cold and was given Cefteram pivoxil (CFTM-PI) for four days. When she arrived at another hospital, she had hypoglycemia (blood glucose 1.1 mmol/L), hepatic dysfunction (AST 85 IU/L, ALT 55 IU/L, LDH 402 IU/L), and mild hyperammoniemia (NH3 84 μmol/L). Urinary ketones were not detected. Brain MRI and cerebrospinal fluid were normal. She was suspected of being affected by a Reye-like syndrome and transferred to Gifu University Hospital.

On admission, her height was 72 cm (−1.5S.D.) and her weight was 10 kg (+0.73S.D.). She had a fever (38.3°C) and exhibited lethargy. Physical examination revealed mild hepatomegaly. A laboratory test showed AST 382 IU/L, ALT 441 IU/L, LDH 557 IU/L, PT 31%, NH3 84 μmol/L, and blood glucose 4.7 mmol/L.

We tentatively diagnosed her as having a Reye-like syndrome and treated her with intravenous glucose. Her consciousness level became clear on the 4th hospital day and she started oral intake of food. An abdominal CT scan still showed hepatomegaly and a fatty liver (20HU) on the 6th hospital day. The finding of cardiac ultrasonography was normal. Urinary organic acid analysis during the hypoglycemic condition showed hypoketotic dicarboxylic aciduria. The initial measurements of serum free carnitine and acyl-

Table 1. Time-course of serum and urinary acylcarnitine levels measured by tandem MS.

	Day	- 1	3	5	7	9	13
Serum (μmol/L)	range						
C0	10 - 55	2.98	12.70	40.75	24.31	18.49	58.22
C2	4 - 60	2.25	3.85	14.87	20.15	8.37	14.8
C8	- 1.0	0.035	0.024	0.088	0.058	0.073	0.10
C8DC	- 0.25	0.035	0.046	0.12	0.89	0.97	0.063
C10	- 0.8	0.055	0.062	0.25	0.12	0.17	0.21
C10DC	- 0.1	0.063	0.12	0.24	0.33	0.53	0.19
C12:1	- 0.2	0.038	0.038	0.18	0.15	0.15	0.091
C12DC	- 0.05	0.053	0.064	0.19	0.14	0.27	0.054
C14:1	- 0.1	0.075	0.16	0.47	0.58	0.68	0.18
C16	- 0.5	1.01	1.29	2.99	4.45	8.07	2.56
C18	- 0.3	0.49	0.65	1.46	1.67	3.07	0.99
C18:1	- 0.46	1.50	1.84	4.21	6.09	10.03	3.62
C18:2	- 0.3	0.46	0.67	1.47	1.43	2.05	0.98
(C16+C18:1)/C2	- 0.36	1.12	0.81	0.48	0.52	2.16	0.42
C total		12.35	26.74	86.07	84.99	67.46	85.52
Urine (μmol/mmol Cr)	range*						
C0	5.67 - 56.09	0.61	1.31	82.33	37.85	45.95	329.15
C2	6.87 - 60.48	0.56	0.02	25.44	128.00	41.83	53.58
C4	0.07 - 0.74	0.31	0.47	0.92	0.47	1.38	2.32
C6	0.04 - 0.48	0.18	0.09	0.21	0.22	0.61	0.23
C6DC		1.25	1.34	1.63	15.69	83.33	2.93
C8	0.05 - 0.39	0.00	0.02	0.33	0.98	1.33	0.62
C8DC		0.25	0.52	0.83	23.90	122.99	1.11
C10	0.03 - 0.36	0.05	0.06	0.11	2.66	1.76	0.12
C10DC		0.11	0.02	0.10	0.75	4.03	0.08
C12DC		0.00	0.02	0.01	0.23	1.52	0.01
C16	0.05 - 1.55	0.04	0.02	0.02	0.18	0.63	0.08
C total		4.75	6.86	122.16	226.51	344.91	408.34

* Reference values for urine acylcarnitines were obtained from data reported by Mueller et al. (2003) (10th - 90th percentile)

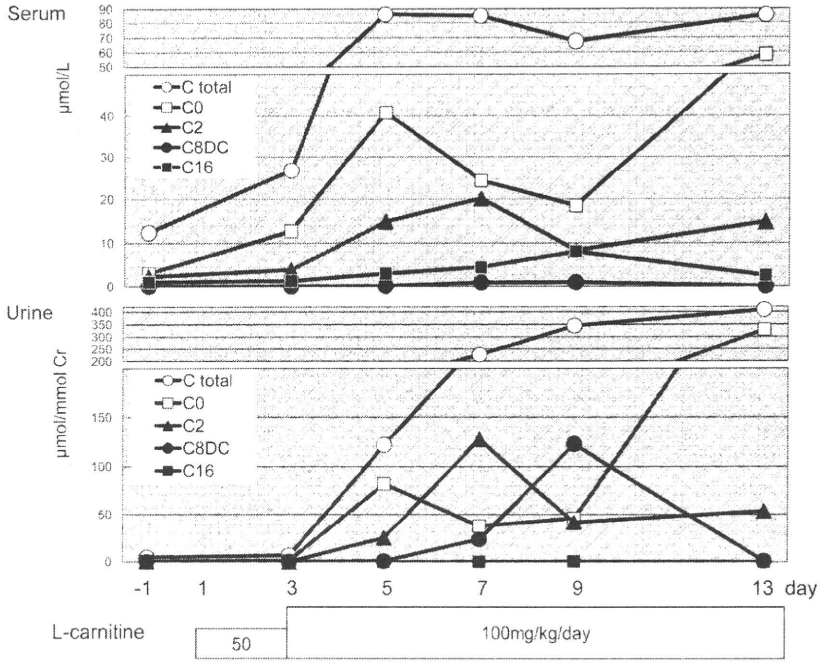

Fig. 1. Time-course of serum and urinary acylcarnitine levels measured by tandem MS. The levels of representative acylcarnitines are shown. The first day of L-carnitine supplementation is designated as day 1. Urinary carnitines were assayed using the first urine in the morning.

carnitine fractions by the enzymatic cycling method were 9.5 and 5.9 μmol/L, respectively. The initial serum acylcarnitine profile (Table 1) showed a very low free carnitine level and relatively high long-chain acylcarnitine levels. This profile was compatible with the secondary carnitine deficiency due to CPT2 or translocase deficiency.

After confirmation of the carnitine deficiency, we supplied her with L-carnitine orally from the 15th hospital day (day 1 in the Table 1 and Fig. 1) at a dose of 50 mg/kg/day for the first two days and 100 mg/kg/day from day 3. Blood and urinary samples were obtained before plus 3, 5, 7, 9 and 13 days after L-carnitine supplementation. During carnitine supplementation, the patient had continuous intravenous glucose infusion of 2.5 mg/kg/min until day 11. We analyzed the serum and urinary acylcarnitines by tandem mass analysis, as previously reported (Mueller et al. 2003; Kobayashi et al. 2007a,b). Table 1 shows details of the analyses. Fig. 1 shows the changing patterns of free carnitine (C0), acetyl-carnitine (C2), C8DC representing medium-chain dicarboxylic acylcarnitines, and C16 representing long-chain acylcarnitines in the serum and urine. Urinary excretion of C0 and acylcarnitines remained at very low levels on day 3. Sequential peaks of free carnitine (day 5), acetylcarnitine (day 7), and long-chain acylcarnitines (day 9) were found in the serum, which corresponded to peaks of free carnitine, acetylcarnitine, and dicarboxylic medium-chain acylcarnitines in the urine.

The fatty liver and hepatomegaly improved as judged by an abdominal CT scan on the 26th hospital day (day 13). Informed consent for a skin biopsy, enzyme assay, and DNA was obtained from the parents. CPT2 activity in the patient's fibroblasts was 0.18 nmol/min/mg of protein (3 controls; 0.82, 1.27, and 1.26 nmol/min/mg of protein), confirming the diagnosis of CPT2 deficiency.

Now the patient is 4 years of age. After carnitine supplementation, she did not experience hypoglycemia at all. She is being treated with 1,000 mg L-carnitine/day (current body weight 19.8 kg). Her growth and development are within normal ranges. She had some rhabdomyolysis attacks (the highest CK recorded was 16,769 IU/L) during a febrile illness even after L-carnitine supplementation.

Discussion

The diagnosis of CPT2 deficiency was first suspected by the data on urinary organic acid analysis and acylcarnitine analysis and was confirmed by enzyme assay using fibroblasts. Our patient is a compound heterozygote of a previously reported E174K mutation from the father and an unknown mutation from the mother which was not detected by exon sequencing. According to an in vitro expression analysis of mutant CPT2 cDNAs carrying E174K, the mutant E174K protein was present as much as a wild type protein and retained 10% residual CPT2 activity (Wataya et al. 1998). This "mild" mutation from the father, together with possible null mutation from the mother, may result in an infantile form of CPT2 deficiency.

Initially, she developed secondary carnitine deficiency. Chronic administration of pivalate-conjugated antibiotics is

a major cause of secondary carnitine deficiency even in healthy children (Stanley 2004). Ten days before the onset of the Reye-like syndrome, she had a cold and was given Cefteram pivoxil (CFTM-PI) for four days. The initial serum acylcarnitine profile showed no elevation of hydroxy-C5 carnitine, nor of pivaloylcarnitine. While the antibiotic might have contributed to secondary carnitine deficiency in part, the acute attack with fasting was more likely the course of the low carnitine in the patient at presentation.

The time-course changes in the serum and urinary acylcarnitine levels after L-carnitine supplementation were studied. These changing profiles suggest that accumulated and potentially toxic long-chain acylcarnitines in the mitochondria were eliminated from the body by day 13. The majority of accumulated long-chain acylcarnitines in the mitochondria may be eliminated by the following steps: 1) a large amount of accumulated long-chain acylcarnitines should be transferred from the mitochondrial matrix by carnitine acylcarnitine translocase if there is a sufficient amount of free carnitine outside of the mitochondrial matrix; 2) then peroxisomal beta-oxidation reduces the chain length of such accumulated fatty acids; 3) the resultant medium-chain fatty acids can be catalyzed in the mitochondria, or further ω-oxidized into dicarboxylic acids in the microsomes; 4) these medium-chain DC and their carnitine conjugates can be excreted into the urine efficiently. It took several days for oral L-carnitine administration to increase the serum carnitine levels, probably because the intracellular stores were depleted and it took several days for them to be replenished. Thereafter, the administration increased the excretion of abnormal acylcarnitines, some of which had probably accumulated within the tissues.

It is noteworthy that the acetylcarnitine in both the serum and the urine was a predominant acylcarnitine on day 7 (Fig. 1). Elevation of acetylcarnitine in the serum and urine indicates the presence of enough acetyl-CoA in the mitochondria and the availability of acetyl-CoA for carnitine acyltransferase reactions in the cells, and might account for the increased beta-oxidation rates upon L-carnitine therapy (Fontaine et al. 1996). In general, acetylcarnitine is a major acylcarnitine in healthy controls and is regarded as a marker of undisturbed beta-oxidation (Costa et al. 1998). Since CPT2-deficient patients have beta-oxidation restrictions of long-chain acyl-CoA, L-carnitine supplementation may increase beta-oxidation of medium-chain acyl-CoAs, which could be supplied via peroxisomal beta-oxidation of long-chain acyl-CoA.

Carnitine supplementation in the treatment of long-chain beta-oxidation defects is still controversial. In patients with a defect in the mitochondrial beta-oxidation spiral, when a preceding L-carnitine deficiency is normalized, and transport into the mitochondria of long-chain fatty acids is also normalized, acyl-CoAs accumulate instead of being oxidized by the defective reaction and, consequently, in such cases, free CoA is depleted in the mitochondria (Yoshino et al. 2003). This may be true in beta-oxidation defects such as very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency and trifunctional protein deficiency. Studies on VLCAD-deficient mice suggested carnitine supplementation results in the induction of acylcarnitine production in various tissues and significant accumulation of potentially toxic intermediate acylcarnitines in tissues (Liebig et al. 2006; Primassin et al. 2008). However, blockage of the CPT2 step causes the accumulation of long-chain acylcarnitines but does not primarily cause the accumulation of intermediate CoA esters in beta-oxidation.

Acknowledgment

This study was supported in part by Health and Labour Science Research Grants for Research on Children and Families from The Ministry of Health, Labour and Welfare of Japan.

References

Bonnefont, J.P., Demaugre, F., Prip-Buus, C., Saudubray, J.M., Brivet, M., Abadi, N. & Thuillier, L. (1999) Carnitine palmitoyltransferase deficiencies. *Mol. Genet. Metab.*, **68**, 424-440.

Bonnefont, J.P., Djouadi, F., Prip-Buus, C., Gobin, S., Munnich, A. & Bastin, J. (2004) Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. *Mol. Aspects Med.*, **25**, 495-520.

Costa, C.G., Dorland, L., de Almeida, I.T., Jakobs, C., Duran, M. & Poll-The, B.T. (1998) The effect of fasting, long-chain triglyceride load and carnitine load on plasma long-chain acylcarnitine levels in mitochondrial very long-chain acyl-CoA dehydrogenase deficiency. *J. Inherit. Metab. Dis.*, **21**, 391-399.

Demaugre, F., Bonnefont, J.P., Colonna, M., Cepanec, C., Leroux, J.P. & Saudubray, J.M. (1991) Infantile form of carnitine palmitoyltransferase II deficiency with hepatomuscular symptoms and sudden death. Physiopathological approach to carnitine palmitoyltransferase II deficiencies. *J. Clin. Invest.*, **87**, 859-864.

Fontaine, M., Briand, G., Vallée, L., Ricart, G., Degand, P., Divry, P., Vianey-Saban, C. & Vamecq, J. (1996) Acylcarnitine removal in a patient with acyl-CoA beta-oxidation deficiency disorder: effect of L-carnitine therapy and starvation. *Clin. Chim. Acta*, **252**, 109-122.

Hug, G., Bove, K.E. & Soukup, S. (1991) Lethal neonatal multiorgan deficiency of carnitine palmitoyltransferase II. *N. Engl. J. Med.*, **325**, 1862-1864.

Kobayashi, H., Hasegawa, Y., Endo, M., Purevsuren, J. & Yamaguchi, S. (2007a) A retrospective ESI-MS/MS analysis of newborn blood spots from 18 symptomatic patients with organic acid and fatty acid oxidation disorders diagnosed either in infancy or in childhood. *J. Inherit. Metab. Dis.*, **30**, 606.

Kobayashi, H., Hasegawa, Y., Endo, M., Purevsuren, J. & Yamaguchi, S. (2007b) ESI-MS/MS study of acylcarnitine profiles in urine from patients with organic acidemias and fatty acid oxidation disorders. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **855**, 80-87.

Liebig, M., Gyenes, M., Brauers, G., Ruiter, J.P., Wendel, U., Mayatepek, E., Strauss, A.W., Wanders, R.J. & Spiekerkoetter, U. (2006) Carnitine supplementation induces long-chain acylcarnitine production—studies in the VLCAD-deficient mouse. *J. Inherit. Metab. Dis.*, **29**, 343-344.

Longo, N., Amat di San Filippo, C. & Pasquali, M. (2006) Disorders of carnitine transport and the carnitine cycle. *Am. J. Med. Genet. C. Semin. Med. Genet.*, **142C**, 77-85.

Mueller, P., Schulze, A., Schindler, I., Ethofer, T., Buehrdel, P. & Ceglarek, U. (2003) Validation of an ESI-MS/MS screening

method for acylcarnitine profiling in urine specimens of neonates, children, adolescents and adults. *Clin. Chim. Acta*, **327**, 47-57.

Primassin, S., Ter Veld, F., Mayatepek, E. & Spiekerkoetter, U. (2008) Carnitine supplementation induces acylcarnitine production in tissues of very long-chain acyl-CoA dehydrogenase-deficient mice, without replenishing low free carnitine. *Pediatr. Res.*, **63**, 632-637.

Stanley, C.A. (2004) Carnitine deficiency disorders in children. *Ann. N.Y. Acad. Sci.*, **1033**, 42-51.

Wataya, K., Akanuma, J., Cavadini, P., Aoki, Y., Kure, S., Invernizzi, F., Yoshida, I., Kira, J., Taroni, F., Matsubara, Y. & Narisawa, K. (1998) Two CPT2 mutations in three Japanese patients with carnitine palmitoyltransferase II deficiency: functional analysis and association with polymorphic haplotypes and two clinical phenotypes. *Hum. Mutat.*, **11**, 377-386.

Yoshino, M., Tokunaga, Y., Watanabe, Y., Yoshida, I., Sakaguchi, M., Hata, I., Shigematsu, Y., Kimura, M. & Yamaguchi, S. (2003) Effect of supplementation with L-carnitine at a small dose on acylcarnitine profiles in serum and urine and the renal handling of acylcarnitines in a patient with multiple acyl-coenzyme A dehydrogenation defect. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **792**, 73-82.

ARTICLE IN PRESS

BBADIS-63241; No. of pages: 6; 4C: 5

Biochimica et Biophysica Acta xxx (2011) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbadis

Clinical and molecular characterization of five patients with succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency

Toshiyuki Fukao [a,b,*], Jörn Oliver Sass [c], Petri Kursula [d,e], Eva Thimm [f], Udo Wendel [f], Can Ficicioglu [g], Kamel Monastiri [h], Nathalie Guffon [i], Ivo Barić [j], Marie-therese Zabot [k], Naomi Kondo [a]

[a] Department of Pediatrics, Graduate School of Medicine, Gifu University, Gifu, Gifu 501-1194, Japan
[b] Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences, Gifu University, Gifu, Gifu 501-1194, Japan
[c] Labor für Klinische Biochemie und Stoffwechsel, Zentrum für Kinder-und Jugendmedizin, Universitätsklinikum Freiburg, 79106 Freiburg, Germany
[d] Department of Biochemistry, University of Oulu, Oulu, Finland
[e] Centre for Structural Systems Biology (CSSB-HZI), German Electron Synchrotron (DESY), Hamburg, Germany
[f] Metabolic unit, University Children's Hospital, Düsseldorf, Germany
[g] Section of Metabolic Disease, The Children's Hospital of Philadelphia, University of Pennsylvania, Philadelphia, PA, USA
[h] Service de Réanimation and Médecine Néonatale, Centre de Maternité and de Néonatologie de Monastir, Monastir, Tunisia
[i] Hopital Femme Mere Enfant, Centre de Reference des Maladies Hereditaires, du Metabolisme, 69677Bron Cedex, France
[j] Department of Pediatrics, University Hospital Center and School of Medicine, Zagreb, Croatia
[k] Centre de Biotechnologie Cellulaire, CBE-GHC, Hospices Civils de Lyon, Lyon, France

ARTICLE INFO

Article history:
Received 8 December 2010
Received in revised form 26 January 2011
Accepted 28 January 2011
Available online xxxx

Keywords:
Ketoacidosis
OXCT
Mutation
SCOT
Succinyl-CoA:3-ketoacid CoA transferase
Structure–function analysis

ABSTRACT

Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency is an inborn error of ketone body metabolism and causes episodic ketoacidosis. We report clinical and molecular analyses of 5 patients with SCOT deficiency. Patients GS07, GS13, and GS14 are homozygotes of S405P, L327P, and R468C, respectively. GS17 and GS18 are compound heterozygotes for S226N and A215V, and V404F and E273X, respectively. These mutations have not been reported previously. Missense mutations were further characterized by transient expression analysis of mutant cDNAs. Among 6 missense mutations, mutants L327P, R468C, and A215V retained some residual activities and their mutant proteins were detected in immunoblot analysis following expression at 37 °C. They were more stable at 30 °C than 37 °C, indicating their temperature sensitive character. The R468C mutant is a distinct temperature sensitive mutant which retained 12% and 51% of wild-type residual activities at 37 and 30 °C, respectively. The S226N mutant protein was detected but retained no residual activity. Effects of missense mutations were predicted from the tertiary structure of the SCOT molecule. Main effects of these mutations were destabilization of SCOT molecules, and some of them also affected catalytic activity. Among 5 patients, GS07 and GS18 had null mutations in both alleles and the other three patients retained some residual SCOT activities. All 5 developed a first severe ketoacidotic crisis with blood gas pH <7.1, and experienced multiple ketoacidotic decompensations (two of them had seven such episodes). In general, the outcome was good even following multiple ketoacidotic events. Permanent ketosis or ketonuria is considered a pathognomonic feature of SCOT deficiency. However, this condition depends not only on residual activity but also on environmental factors.

1. Introduction

Ketone bodies, produced mainly in the liver, are an important source of energy for extrahepatic tissues [1]. Succinyl-CoA: 3-ketoacid CoA transferase (SCOT; EC 2.8.3.5, gene symbol OXCT1) is a mitochondrial homodimer essential for ketone body utilization. SCOT catalyzes acetoacetate activation to acetoacetyl-CoA in mitochondria. The human OXCT1 gene is mapped to 5p13 and consists of 17 exons [2,3]. Human SCOT cDNA encodes a precursor subunit of 520 amino acids.

Patients with SCOT deficiency (OMIM 245050) experience episodic ketoacidosis and are usually asymptomatic between episodes. Fewer than 30 affected individuals are known [2–22]. Urinary organic acid analysis and acylcarnitine analysis show non-specific profiles in this disorder. Hence, in vitro methods of diagnosis, such as enzyme assay and mutation analysis, are essential for the definite diagnosis. Permanent ketosis or ketonuria is a pathognomonic feature of this disorder but is not always present [17,20,22]. We previously identified 11 mutations of the OXCT1 gene [6,8–14] in 12 SCOT-deficient families.

Recently, the human SCOT tertiary structure has become available (PDB entry 3DLX). This has enabled us to evaluate effects of missense

* Corresponding author at: Medical Information Sciences Division, United Graduate School of Drug Discovery and Medical Information Sciences; Department of Pediatrics, Graduate School of Medicine, Gifu University, 1-1 Yanagido, Gifu, Gifu 501-1194, Japan. Tel.: +81 58 230 6386; fax: +81 58-230 6387.
E-mail address: toshi-gif@umin.net (T. Fukao).

0925-4439/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbadis.2011.01.015

Please cite this article as: T. Fukao, et al., Clinical and molecular characterization of five patients with succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.01.015

mutations more precisely than homology modeling using porcine SCOT structure [20].

We herein describe 5 SCOT-deficient patients and characterize their mutations by transient expression analysis of mutant cDNAs and discuss the mutation sites on the tertiary structure of human SCOT.

2. Patients, materials and methods

2.1. Patients

GS07: Clinical findings of GS07 have been reported previously [11]. Briefly, he presented with two episodes of ketoacidosis during infections at 17 and 25 months of age. He was followed until age 6 years without any further episodes.

GS13: Some aspects of GS13 have already been published [14]. Although parents are not known to be related, the family originates from a small socially isolated area, making consanguinity not unlikely. After an initial severe crisis at age 6 months, the girl had three additional, but milder crises during infancy. After 6 h of fasting during a test performed after the first crisis, her blood pH was 7.42, HCO_3 12.5 mmol/L and BE −9.1 mmol/L, with high serum ketones 3.55 mmol/L and low FFA of 0.29 mmol/L. At the age of 12.5 years she is now doing well, attending a regular school. She usually takes her last meal at about 10 p.m. and takes first morning meal in school at about 9 to 10 a.m. without any signs or symptoms. Acid-base balance was checked three times at about 8 a.m. and was always normal.

GS14: This female patient of 21 years of age was born to consanguineous parents in October 1988. She had many hospitalizations in the pediatric ward for episodes of hypoglycemia. Her first hospitalization was at 19 months for seizures and coma. On physical exam she had deep respiration. Her blood glucose was 1.3 mmol/L. She had severe metabolic acidosis with pH 6.93, HCO_3 6 mmol/L and ketonuria 3+. She had six similar episodes of hypoglycemia with ketoacidosis without seizures, usually after episodes of infections. Her neuro-developmental status was normal. She had persistent ketonuria between episodes of decompensation.

GS17: She is a Caucasian girl born to non-consanguineous parents at full-term with a birth weight of 2230 g. She had low blood glucose levels of 1.6 mmol/L on the first day of life and received intravenous glucose for 1–2 days. She was discharged on day 5 of life. She was well until 3 years of age when she developed tachypnea and lethargy following gastroenteritis. Her blood pH was 6.99 with BE −25 mmol/L and her blood glucose was 7.5 mmol/L. Urine analysis showed massive ketones. She was intubated and admitted to the intensive care unit. She responded to intravenous glucose and bicarbonate and was discharged after 7 days. Six months later she was readmitted to a local hospital with mild lethargy, tachypnea and ketoacidosis which developed during a febrile upper respiratory infection. The patient's mother reported that the patient has had trace to moderate ketonuria, even when she was in good health. SCOT deficiency was confirmed by an enzyme assay using fibroblasts. The patient has not had any more episodes of ketoacidosis for 6 years. She is doing well and receives a mildly protein-restricted diet (2.0 g/kg/day), avoids prolonged fasting and adheres to "sick day" precautions such as increasing calories from carbohydrates in her diet and intravenous glucose as needed.

GS18: The male patient, first child of healthy non-consanguineous parents from Vietnam, was born at 39 weeks with a birth weight of 3390 g. After a normal clinical presentation during the first days of life he was readmitted to hospital at the age of 3 days with polypnea. Biochemically he presented with severe metabolic acidosis (pH 7.08, pCO_2 25 mmHg, BE −22.6 mmol/l) and pronounced ketonuria. Beside ketones, metabolic screening revealed unremarkable urinary organic acids. With intravenous fluid with sodium bicarbonate the patient recovered within hours. During the first year of life the patient was hospitalized three times because of episodes of severe ketoacidosis (minimal pH 6.98, pCO_2 15 mmHg, BE −28 mmol/l). At the age of 1 year, SCOT deficiency was confirmed by an enzyme assay using his lymphocytes and platelets. He was on treatment that consisted of avoidance of prolonged fasting and moderate protein restriction (1.5 g/kg/d). Subsequently the patient has had three more severe episodes of ketoacidosis in the course of intercurrent diseases. Otherwise, he has permanent mild ketonuria. At his present age of 10 years psychomotor and physical development are normal.

Table 1 summarizes the clinical presentations and laboratory data of these 5 SCOT-deficient patients. This study has been approved by The Ethical Committee of Graduate School of Medicine, Gifu University.

2.2. Enzyme assay and immunoblot analysis

Assays for acetoacetyl-CoA thiolase and for SCOT were performed as described [7,23], using acetoacetyl-CoA as a substrate and measuring its disappearance spectrophotometrically.

2.3. Mutation analysis

Total RNA was purified from peripheral blood mononuclear cells with an ISOGEN kit (Nippon Gene, Tokyo, Japan). RT-PCR was as described [2]. Mutations were detected by amplifying cDNA spanning the full-length coding sequence, and sequencing more than 5 clones.

Genomic DNA was purified with a Sepa Gene kit (Sanko Junyaku, Tokyo, Japan). Mutation analysis at the genomic level was done by PCR for each exon and its intron boundaries (at least 20 bases from the exon/intron boundaries for both directions) followed by direct sequencing [3].

2.4. Construction of eukaryote transient expression vectors

Wild-type full-length SCOT cDNAs [3] were subcloned into the pTZ18U and pCAGGS eukaryote expression vectors [24] and designated the pTscotWild-type and pCAGGSscotWild-type, respectively. Mutations were introduced into the pTscotWild-type using a QuikChange Site-Directed Mutagenesis kit (Stratagene, La Jolla CA), confirmed by sequencing, and then transferred into pCAGGS.

2.5. Transient expression analysis

Wild-type and mutant SCOT expression vectors (4 μg) were first transfected using Lipofectamine 2000 (GIBCO BRL Invitrogen Inc., Carlsbad, CA) in ~10^5 SV40-transformed SCOT-deficient fibroblasts of GS01[2]. One microgram of the cytosolic acetoacetyl-CoA thiolase (CT)-expressing vector, pCAGGSct [25], was cotransfected to monitor transfection efficiency. Transfection was done at 37 °C for 24 h was followed by a further 48-h incubation at 37 or 30 °C. The cells were harvested and stored at −80 °C until SCOT and CT activities were assayed. Immunoblot was done using a mixture of anti-[human SCOT] antibody and anti-[human CT] antibody as the first antibody [26]. The quantity of mutant protein was estimated densitometrically, and was compared to the signal intensities of serially diluted samples of the wild-type SCOT protein.

2.6. Tertiary structural model of human SCOT

To analyze the putative structural implications of the SCOT mutations, the recently determined crystal structure from the Structural Genomics Consortium (PDB entry 3DLX) of human SCOT was taken as a starting point. Prior to the analysis, the structure was subjected to further refinement in PHENIX [27] and COOT [28], including the addition of missing side chains and rebuilding of the solvent network. The figures describing the structural details were prepared with PyMOL.

Please cite this article as: T. Fukao, et al., Clinical and molecular characterization of five patients with succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.01.015

Table 1
Clinical presentation and mutation of SCOT-deficient cases.

GS number	Nationality	Sex	Consanguinity	Affected siblings	First ketoacidotic crisis						Persistent ketonuria	Frequency of crisis	Present age	Prognosis	Gene mutation		SNP			
					Onset	Symptom	Blood gas pH	HCO_3	BE	Glucose					Paternal	Maternal	rs75134564	rs1136453		
																	c.173C>T(T58M)	c.315 G>C(S105S)	c.1497 A>G(Q499Q)	
GS07	France	M	+	–	1y5m	Polypnea, lethargy	6.92	4		na	+	2	(6y)	NP	c.1213T>C (S405P)	c.1213T>C (S405P)	C/C	C/C	G/G	
GS13	Croatia	F	*	–	6 m	Appetite loss, coma	6.90	3.2	−27	7.8	–	2	12y	NP	c.980T>C (L327P)	c.980T>C (L327P)	C/C	C/C	G/G	
GS14	Tunisia	F	+	–	1y7m	Seizure, coma	6.93	6		1.3	+	7	21y	NP	c.1402C>T (R468C)	c.1402C>T (R468C)	C/C	C/C	G/G	
GS17	USA	F	–	–	3y	Polypnea	6.99	5	−25	7.5	+	2	9y	NP	c.644C>T (A215V)	c.677G>A (S226N)	C/C	C/C	G/G	
GS18	Germany**	F	–	+	3d	Polypnea, lethargy	7.08		−22.6	9.1	+	5	10y	NP	c.817G>T (E273X)	c.1210G>T (V404F)	C/T	G/G	A/A	

NP: nothing particular.
* Possible consanguinity.
** GS18 is Vietnamese.

3. Results and discussion

3.1. Enzyme assay

Enzyme assay data for 4 patients are shown in Table 2. All four patients' fibroblasts presented with decreased SCOT activity, whereas they had a potassium-ion activated acetoacetyl-CoA thiolase activity which was a specific character of mitochondrial acetoacetyl-CoA thiolase (T2). In immunoblot analysis, SCOT protein was scarcely detected in these patients' cells, whereas T2 protein was clearly detected (data not shown). Lymphocytes and platelets from GS18 had no apparent SCOT activity (data not shown). These results confirmed the diagnosis of SCOT deficiency in the 5 patients.

3.2. Mutation analysis

Both genomic mutation analysis and cDNA analysis were done in all the cases except for GS18 of whom RNA was not available. The results of mutation analyses are shown in Table 1. Three patients with definite or possible consanguinity had homozygous mutations (c.1213T>C (S406P) in GS07; c.980T>C (L327P) in GS13; c.1402C>T (R468C) in GS14). GS17 is a compound heterozygote of c.644C>T (A215V) from the father and c.677G>A (S266N) from the mother. GS18 is also a compound heterozygote of c.817G>T (E273X) from the father and c.1210G>T (V404F) from the mother. We also detected three single nucleotide polymorphisms. Among them, c.173C>T (T58M) (rs75134564) was previously identified in a Japanese patient (GS02) and demonstrated not to reduce enzyme activity [13].

3.3. Transient expression analysis of mutant cDNAs

We performed transient expression analysis of wild-type and mutant cDNAs in SCOT-deficient SV40-transformed fibroblasts. Following expression of SCOT cDNAs for 48 h at either 37 or 30 °C, an enzyme assay and immunoblots were performed (Fig. 1). The transfection of wild-type SCOT cDNA produced high SCOT activity, whereas that of mock cDNA produced no demonstrable enzyme activity at any temperature. Among 6 missense mutations, S226N, V404F, and S405P did not retain residual SCOT activity. The A215V, L327P and R468C mutants retained detectable residual activities, 3.5, 4.7 and 12% of the wild-type value, respectively, in expression at 37 °C. Their relative residual SCOT activities to wild-type in expression at 30 °C were 3- to 4-fold higher than those in expression at 37 °C. In particular, R468C mutants retained a 51% activity of wild-type value in the expression at 30 °C. In immunoblot analysis, V404F and S405P protein was not detected in expression at 30 and 37 °C. S226N protein was clearly detected in the expression at 30 °C without any detectable residual activity, indicating that S226N protein was an inactive protein. The relative amount of the A215V, L327P, and R468C mutant proteins, as compared to the wild-type, was estimated to be 30%, 30%, and 50%, respectively, in expression at 30 °C. These proteins were more stable at 30 °C than at 37 °C. Specific activities (activity/protein) of A215V, L327P, and R468C mutants could be calculated to about 50%, 50%, and 100% that of wild-type, respectively.

Table 2
Enzyme assay using fibroblasts.

	Acetoacetyl-CoA thiolase			SCOT	SCOT/+K^+
	−K^+	+K^+	+K^+/−K^+		
GS07	5.9	12.9	2.2	1.9	0.2
GS13	4.3	9.1	2.1	0.8	0.1
GS14	3.6	7.3	2.0	1.2	0.2
GS17	7.0	14.9	2.1	1.2	0.1
Controls ($n=5$)	5.0 ± 0.7	10.8 ± 0.9	2.2 ± 0.3	6.7 ± 2.1	0.6 ± 0.2

Enzyme activity is expressed as nmol/min/mg protein.
Fibroblasts from GS18 were not available.

Please cite this article as: T. Fukao, et al., Clinical and molecular characterization of five patients with succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.01.015

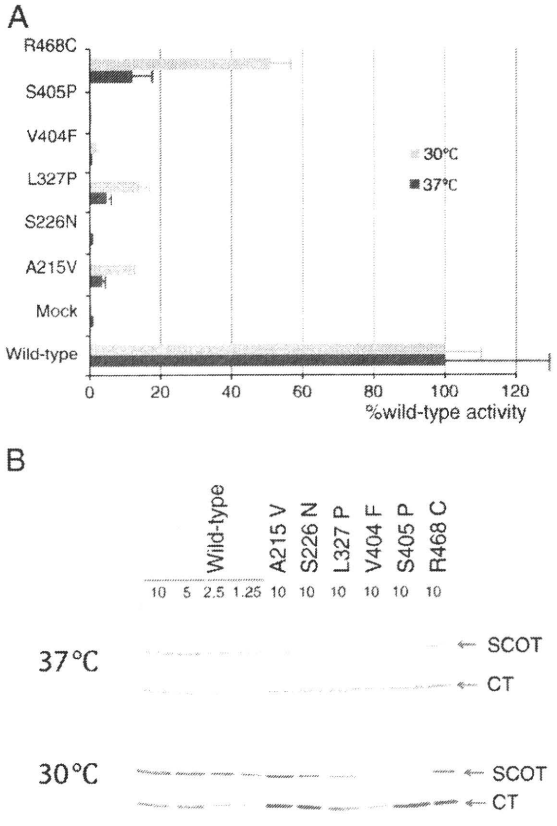

Fig. 1. Transient expression results for wild-type and mutant SCOT cDNAs. Wild-type and mutant SCOT expression vectors (4 μg) were transfected together with 1 μg of the cytosolic acetoacetyl-CoA thiolase (CT)-expressing vector, pCAGGSct, to SV40-transformed fibroblasts of GS01 of which the mutation is S283X/S283X. Transient expression was done 37 and 30 °C. Mock, transfection of 1 μg of pCAGGSct and 4 μg of pCAGGS vectors without insert. (A) SCOT activities relative to those in wild-type transfection are shown. The mean values are displayed together with the SD of three independent experiments. (B) Immunoblots for SCOT and CT are shown. The protein amounts applied are shown above the lanes. The first antibody was a mixture of an anti-CT (cytosolic thiolase) antibody and anti-SCOT antibody. The positions of the bands for CT and SCOT are indicated by arrows.

3.4. Tertiary structural model of human SCOT and mutations

A number of mutations have been characterized for SCOT deficiency, and several of them have been structurally analyzed before [3,20,22], based on homology models of human SCOT, made with the help of the pig SCOT crystal structure [20]. Fig. 2A shows a dimer of human SCOT, with presently and previously identified mutations highlighted on the SCOT monomer in Fig. 2B. It is noteworthy that most of the mutations are located around two 'hot-spots' in 3D space; these areas correspond to a small beta sandwich domain in the N-terminal lobe, and a larger beta sandwich structure close to the C terminus. Sporadic mutations are also seen closer to the active site cavity.

The mutation A215V involves the residue A215, which in the wild-type protein is in the middle of a beta sheet, pointing inwards into the protein. The terminal carbon atom of A215 is only 3.6 Å away from the terminal methyl group carbons of L269 in an opposing beta sheet. Thus, even a small valine residue cannot be incorporated into this position without structural strain and changes. The position is located at a small beta sandwich domain involved in SCOT dimer formation. A215 is, furthermore, in the very close vicinity of the previously characterized SCOT mutations G219E and V221M [3]. These observations on the tertiary structure are in accord with the results that the main mutant effect of A215V is the instability of the mutant protein since the mutant protein amount was 12.5% and 30% relative to wild-type at 37 and 30 °C, respectively.

S226 is located close to the dimerization interface, although not being directly involved in it. The side chain is hydrogen-bonded to the backbone carbonyl of D362 and *via* a buried water molecule to N345; N345 is vicinal to the crucial catalytic residue E344 (Fig. 2D). Thus, the S226N mutation is likely to disturb the structure at least locally, and could also affect the properties of the catalytic site. As expected from the view of tertiary structure, the S226N mutant protein was revealed to be unstable and non-functional protein was detected in transient expression analysis.

L327 locates to an alpha helix on the SCOT surface, close to the active site entrance (Fig. 2D). The side chain of L327 is solvent-exposed and disordered in the crystal. This helix could form part of the CoA substrate-binding site, and a proline mutation in the central part of this helix may both perturb the helical structure and affect the functional mobility of this region, especially since the neighboring residue (326) is also a proline. A main mutant effect of L327P is the instability of the mutant protein since the mutant protein amount was 12.5% and 30% relative to wild-type at 37 and 30 °C, respectively.

V404 locates to the C-terminal beta sandwich domain of SCOT (Fig. 2E). The side chain points inwards, into a tightly packed hydrophobic core between two beta sheets. There clearly is no room for a large Phe residue, as there are several short distances from the side chain of V404 to residues from the opposing beta sheet. S405 is neighboring V404, at the end of a beta strand, preceding a short tight beta turn (Fig. 2E). In light of this, the side chain hydroxyl group is within hydrogen bonding distance from three main-chain NH groups from residues 407–409. Such an arrangement would be completely destroyed upon mutation of residue 405 to proline. As expected from the tertiary structure, these mutant proteins were too unstable to detect in either the 37 or 30 °C expression.

R468 is an exposed residue, present in the beta sheet opposite to that harboring V404 and S405. Its side chain is a central residue in a salt bridge network with E488, R308, E312, K436, and D477 (Fig. 2F). Mutation of the R468 residue will be detrimental to this large hydrophilic region on the surface of the C-terminal domain of SCOT. Since its specific activity is similar to wild-type, R468C does not affect catalytic activity; the active site residues are far from this mutation. The main mutational effect of R468C is also instability of the molecule.

3.5. Clinical phenotypes and genotypes

We reported herein 5 SCOT-deficient patients and their clinical and molecular aspects are summarized in Table 1. They developed the first ketoacidotic episodes from 3 days of age to 3 years of age. The episodes were associated with very severe metabolic acidosis with blood pH ranging from 6.90 to 7.08. They all recovered from the first ketoacidotic crises and were well managed after the diagnosis of SCOT deficiency was made. We previously reported clinical and molecular characters for 12 SCOT-deficient families and now have added to those 5 more.

Permanent ketosis or persistent ketonuria is pathognomonic feature of SCOT deficiency. We, however, previously showed that SCOT-deficient patients with "mild" mutations may have no permanent ketosis. V221M, R268H, or T435N homozygotes and T435N/null mutation compound heterozygotes did not show permanent ketosis or permanent ketonuria [3,17,20,22]. V221M, R268H and T435N mutations retained 10%, 34% and 25%, respectively, relative activity to wild-type in 37 °C expression using the same expression system. In the present study, L327P and R468H mutations retained 4.7% and 12%, respectively, relative activity in 37 °C expression. A L327P homozygote, GS13, did not have permanent ketonuria but a R468H homozygote; GS14 did. To our knowledge, patients with null mutations all showed permanent ketonemia or ketonuria. Hence "mild" mutation with

Please cite this article as: T. Fukao, et al., Clinical and molecular characterization of five patients with succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.01.015

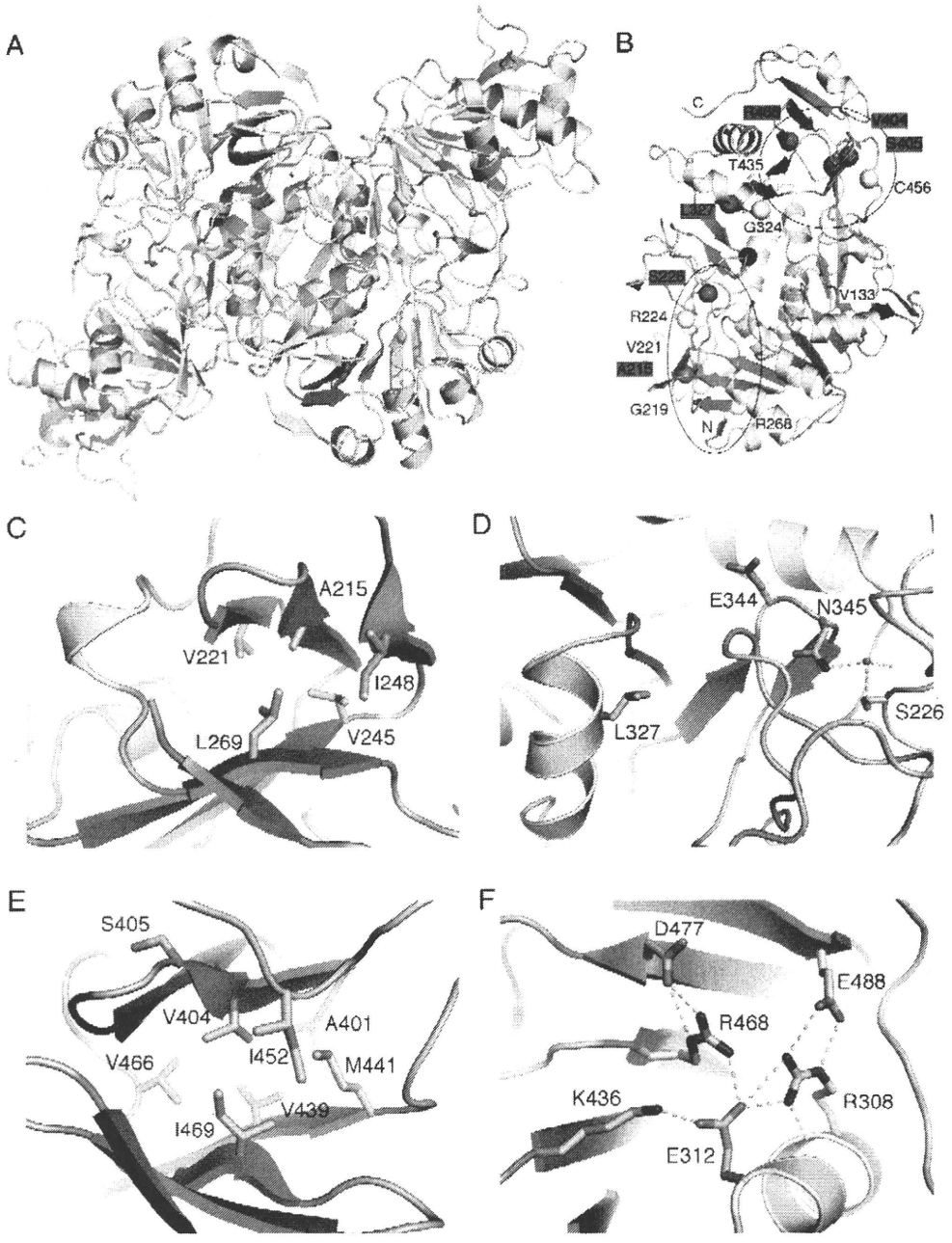

Fig. 2. Mutation sites on the tertiary structure of SCOT monomer. (A) An overall structure of human SCOT dimmer (PDB entry 3DLX). (B) A monomer of human SCOT. The N and C termini are labelled. The two clusters of mutations are indicated by ellipsoids. The positions of the mutations identified in this study are in red, and the ones previously identified are shown in yellow. The position of the catalytically active glutamate residue 344 is marked with a blue sphere. (C) The environment of the A215V mutation in the tightly packed hydrophobic core of the small beta sandwich. The N terminus of the crystal structure is at the bottom front. (D) S226 lies close to the active site, and interacts with N345 via a water-mediated hydrogen bond (green) and by van der Waals interactions. The catalytic residue is E344. L327 also lies close to the entrance of the catalytic cavity and could be involved in substrate binding. (E) V404 and S405 are next to each other, V404 being buried at the hydrophobic core of a beta sandwich unit. S405 interacts with the backbone amides of a tight turn (blue). (F) R468 plays a central role in a salt bridge network linking a beta sheet and a helix.

residual activity may be a necessary condition but not a sufficient condition for the absence of permanent ketonemia or ketonuria. Environmental factors may also affect a clinical phenotype of persistent ketonuria.

Acknowledgment

We thank N. Sakaguchi and K. Murase for technical assistance. We also thank Paul Langman, Ph.D. for his kind assistance with English usage. This study was supported in part by a Grant-in-Aid for Scientific Research (18591148) from the Ministry of Education, Science, Sports and Culture of Japan, by Health and Labour Science Research Grants for Research on Children and Families from The Ministry of Health, Labour and Welfare of Japan and grant 108-1081870-1885 (to I.B.) of the Ministry of Science, Education and Sports, Republic of Croatia, and by the Müller-Fahnenberg-Stiftung Freiburg, Germany (to J.O.S.). PK is an Academy Research Fellow (Academy of Finland).

Please cite this article as: T. Fukao, et al., Clinical and molecular characterization of five patients with succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.01.015

References

[1] G.A. Mitchell, T. Fukao, Chapter 102. Inborn errors of ketone body catabolism, in: C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle (Eds.), Metabolic and molecular bases of inherited disease, 8th edition, McGraw-Hill, Inc, NewYork, 2001, pp. 2327–2356.

[2] S. Kassovska-Bratinova, T. Fukao, X.Q. Song, A.M. Duncan, H.S. Chen, M.F. Robert, C. Perez-Cerda, M. Ugarte, C. Chartrand, S. Vobecky, N. Kondo, G.A. Mitchell, Succinyl CoA: 3-oxoacid CoA transferase (SCOT): human cDNA cloning, human chromosomal mapping to 5p13, and mutation detection in a SCOT-deficient patient, Am. J. Hum. Genet. 59 (1996) 519–528.

[3] T. Fukao, G.A. Mitchell, X.Q. Song, H. Nakamura, S. Kassovska-Bratinova, K.E. Orii, J. E. Wraith, G. Besley, R.J. Wanders, K.E. Niezen-Koning, G.T. Berry, M. Palmieri, N. Kondo, Succinyl-CoA:3-ketoacid CoA transferase (SCOT): cloning of the human SCOT gene, tertiary structural modeling of the human SCOT monomer, and characterization of three pathogenic mutations, Genomics 68 (2000) 144–151.

[4] M. Cornblath, R.L. Gingell, G.A. Fleming, J.T. Tildon, A.T. Leffler, R.A. Wapnir, A new syndrome of ketoacidosis in infancy, J. Pediatr. 79 (1971) 413–418.

[5] J.T. Tildon, M. Cornblath, Succinyl-CoA: 3-ketoacid CoA-transferase deficiency. A cause for ketoacidosis in infancy, J. Clin. Invest. 51 (1972) 493–498.

[6] C. Perez-Cerda, B. Merinero, P. Sanz, A. Jimenez, C. Hernandez, M.J. Garcia, M. Ugarte, A new case of succinyl-CoA: acetoacetate transferase deficiency, J. Inherit. Metab. Dis. 15 (1992) 371–373.

[7] H. Sakazaki, K. Hirayama, S. Murakami, S. Yonezawa, H. Shintaku, Y. Sawada, T. Fukao, H. Watanabe, T. Orii, G. Isshiki, A new Japanese case of succinyl-CoA: 3-ketoacid CoA-transferase deficiency, J. Inherit. Metab. Dis. 18 (1995) 323–325.

[8] C.J. Pretorius, G.G. Loy Son, F. Bonnici, E.H. Harley, Two siblings with episodic ketoacidosis and decreased activity of succinyl-CoA:3-ketoacid CoA-transferase in cultured fibroblasts, J. Inherit. Metab. Dis. 19 (1996) 296–300.

[9] T. Fukao, X.Q. Song, H. Watanabe, K. Hirayama, H. Sakazaki, H. Shintaku, M. Imanaka, T. Orii, N. Kondo, Prenatal diagnosis of succinyl-coenzyme A:3-ketoacid coenzyme A transferase deficiency, Prenat. Diagn. 16 (1996) 471–474.

[10] K.E. Niezen-Koning, R.J. Wanders, J.P. Ruiter, L. Ijlst, G. Visser, W.C. Reitsma-Bierens, H.S. Heymans, D.J. Reijngoud, G.P. Smit, Succinyl-CoA:acetoacetate transferase deficiency: identification of a new patient with a neonatal onset and review of the literature, Eur. J. Pediatr. 156 (1997) 870–873.

[11] M.O. Rolland, N. Guffon, G. Mandon, P. Divry, Succinyl-CoA:acetoacetate transferase deficiency. Identification of a new case; prenatal exclusion in three further pregnancies, J. Inherit. Metab. Dis. 21 (1998) 687–688.

[12] S.E. Snyderman, C. Sansaricq, B. Middleton, Succinyl-CoA:3-ketoacid CoA-transferase deficiency, Pediatrics 101 (1998) 709–711.

[13] X.Q. Song, T. Fukao, H. Watanabe, H. Shintaku, K. Hirayama, S. Kassovska-Bratinova, N. Kondo, G.A. Mitchell, Succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency: two pathogenic mutations, V133E and C456F, in Japanese siblings, Hum. Mutat. 12 (1998) 83–88.

[14] I. Baric, V. Sarnavka, K. Fumic, M. Maradin, D. Begovic, J.P. Ruiter, R.J. Wanders, A new case of succinyl-CoA:acetoacetate transferase deficiency: favourable course despite very low residual activity, J. Inherit. Metab. Dis. 24 (2001) 81–82.

[15] G.T. Berry, T. Fukao, G.A. Mitchell, A. Mazur, M. Ciafre, J. Gibson, N. Kondo, M.J. Palmieri, Neonatal hypoglycaemia in severe succinyl-CoA: 3-oxoacid CoA-transferase deficiency, J. Inherit. Metab. Dis. 24 (2001) 587–595.

[16] N. Longo, T. Fukao, R. Singh, M. Pasquali, R.G. Barrios, N. Kondo, K.M. Gibson, Succinyl-CoA:3-ketoacid transferase (SCOT) deficiency in a new patient homozygous for an R217X mutation, J. Inherit. Metab. Dis. 27 (2004) 691–692.

[17] T. Fukao, H. Shintaku, R. Kusubae, G.X. Zhang, K. Nakamura, M. Kondo, N. Kondo, Patients homozygous for the T435N mutation of succinyl-CoA:3-ketoacid CoA transferase (SCOT) do not show permanent ketosis, Pediatr. Res. 56 (2004) 858–863.

[18] T. Fukao, S. Sakurai, M.O. Rolland, M.T. Zabot, A. Schulze, K. Yamada, N. Kondo, A 6-bp deletion at the splice donor site of the first intron resulted in aberrant splicing using a cryptic splice site within exon 1 in a patient with succinyl-CoA: 3-ketoacid CoA transferase (SCOT) deficiency, Mol. Genet. Metab. 89 (2006) 280–282.

[19] K. Yamada, T. Fukao, G. Zhang, S. Sakurai, J.P. Ruiter, R.J. Wanders, N. Kondo, Single-base substitution at the last nucleotide of exon 6 (c.671G>A), resulting in the skipping of exon 6, and exons 6 and 7 in human succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene, Mol. Genet. Metab. 90 (2007) 291–297.

[20] T. Fukao, P. Kursula, E.P. Owen, N. Kondo, Identification and characterization of a temperature-sensitive R268H mutation in the human succinyl-CoA:3-ketoacid CoA transferase (SCOT) gene, Mol. Genet. Metab. 92 (2007) 216–221.

[21] S. Merron, R. Akhtar, Management and communication problems in a patient with succinyl-CoA transferase deficiency in pregnancy and labour, Int. J. Obstet. Anesth. 18 (2009) 280–283.

[22] T. Fukao, T. Ishii, N. Amano, P. Kursula, M. Takayanagi, K. Murase, N. Sakaguchi, N. Kondo, T. Hasegawa, A neonatal-onset succinyl-CoA:3-ketoacid CoA transferase (SCOT)-deficient patient with T435N and c.658–666dupAACGTGATT p. N220_I222dup mutations in the OXCT1 gene, J. Inherit. Metab. Dis. (2010).

[23] T. Fukao, X.Q. Song, G.A. Mitchell, S. Yamaguchi, K. Sukegawa, T. Orii, N. Kondo, Enzymes of ketone body utilization in human tissues: protein and messenger RNA levels of succinyl-coenzyme A (CoA):3-ketoacid CoA transferase and mitochondrial and cytosolic acetoacetyl-CoA thiolases, Pediatr. Res. 42 (1997) 498–502.

[24] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, Gene 108 (1991) 193–199.

[25] X.Q. Song, T. Fukao, S. Yamaguchi, S. Miyazawa, T. Hashimoto, T. Orii, Molecular cloning and nucleotide sequence of complementary DNA for human hepatic cytosolic acetoacetyl-coenzyme A thiolase, Biochem. Biophys. Res. Commun. 201 (1994) 478–485.

[26] X.Q. Song, T. Fukao, G.A. Mitchell, S. Kassovska-Bratinova, M. Ugarte, R.J. Wanders, K. Hirayama, H. Shintaku, P. Churchill, H. Watanabe, T. Orii, N. Kondo, Succinyl-CoA:3-ketoacid coenzyme A transferase (SCOT): development of an antibody to human SCOT and diagnostic use in hereditary SCOT deficiency, Biochim. Biophys. Acta 1360 (1997) 151–156.

[27] P.D. Adams, P.V. Afonine, G. Bunkoczi, V.B. Chen, I.W. Davis, N. Echols, J.J. Headd, L. W. Hung, G.J. Kapral, R.W. Grosse-Kunstleve, A.J. McCoy, N.W. Moriarty, R. Oeffner, R.J. Read, D.C. Richardson, J.S. Richardson, T.C. Terwilliger, P.H. Zwart, PHENIX: a comprehensive Python-based system for macromolecular structure solution, Acta Crystallogr. D Biol. Crystallogr. 66 (2010) 213–221.

[28] P. Emsley, K. Cowtan, Coot: model-building tools for molecular graphics, Acta Crystallogr. D Biol. Crystallogr. 60 (2004) 2126–2132.

Please cite this article as: T. Fukao, et al., Clinical and molecular characterization of five patients with succinyl-CoA:3-ketoacid CoA transferase (SCOT) deficiency, Biochim. Biophys. Acta (2011), doi:10.1016/j.bbadis.2011.01.015

Journal of Chromatography B, 878 (2010) 1673–1676

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

In vitro probe acylcarnitine profiling assay using cultured fibroblasts and electrospray ionization tandem mass spectrometry predicts severity of patients with glutaric aciduria type 2☆

Mitsuru Endo, Yuki Hasegawa*, Seiji Fukuda, Hironori Kobayashi, Yuka Yotsumoto, Yuichi Mushimoto, Hong Li, Jamiyan Purevsuren, Seiji Yamaguchi

Department of Pediatrics, Shimane University School of Medicine, 89-1 En-ya, Izumo, Shimane 693-8501, Japan

ARTICLE INFO

Article history:
Received 22 November 2009
Accepted 16 March 2010
Available online 20 March 2010

Keywords:
Multiple acyl-CoA dehydrogenase deficiency
In vitro probe assay
Acylcarnitine profile
Clinical severity
ESI/MS/MS

ABSTRACT

Glutaric aciduria type 2 (multiple acyl-CoA dehydrogenase deficiency, MAD) is a multiple defect of mitochondrial acyl-CoA dehydrogenases due to a deficiency of electron transfer flavoprotein (ETF) or ETF dehydrogenase. The clinical spectrum are relatively wide from the neonatal onset, severe form (MAD-S) to the late-onset, milder form (MAD-M). In the present study, we determined whether the in vitro probe acylcarnitine assay using cultured fibroblasts and electrospray ionization tandem mass spectrometry (MS/MS) can evaluate their clinical severity or not. Incubation of cells from MAD-S patients with palmitic acid showed large increase in palmitoylcarnitine (C16), whereas the downstream acylcarnitines; C14, C12, C10 or C8 as well as C2, were extremely low. In contrast, accumulation of C16 was smaller while the amount of downstream metabolites was higher in fibroblasts from MAD-M compared to MAD-S. The ratio of C16/C14, C16/C12, or C16/C10, in the culture medium was significantly higher in MAD-S compared with that in MAD-M. Loading octanoic acid or myristic acid led to a significant elevation in C8 or C12, respectively in MAD-S, while their effects were less pronounced in MAD-M. In conclusion, it is possible to distinguish MAD-S and MAD-M by in vitro probe acylcarnitine profiling assay with various fatty acids as substrates. This strategy may be applicable for other metabolic disorders.

1. Introduction

Fatty acid oxidation disorders (FAODs) potentially cause energy crises that are triggered by stress brought on by starvation or infection, and manifest themselves through nonketotic hypoglycemia, acute encephalopathy, or symptoms similar to those of Reye's syndrome. Among FAODs, medium-chain acyl-CoA dehydrogenase deficiency (MCAD-def) is most common among Caucasian [1], whereas very long-chain acyl-CoA dehydrogenase deficiency (VLCAD-def) and carnitine palmitoyl-CoA transferase 2 deficiency (CPT2-def) are common in Japanese, followed by glutaric aciduria type 2 (multiple acyl-CoA dehydrogenase deficiency: MAD) [2].

In MAD, multiple mitochondrial FAD-dependent dehydrogenases are impaired due to a defect in α- or β-subunits of electron transfer flavoprotein (ETF-α and ETF-β; OMIM 608053 and 130410 respectively) or ETF dehydrogenase (ETF-DH; OMIM 231675) [3,4]. The clinical forms of MAD include the neonatal-onset form (severe form: MAD-S) and the late-onset form (milder form: MAD-M). MAD-S occurs during the neonatal period, and is fatal. MAD-M often becomes symptomatic after infancy, and has episodic symptoms of hypotonia, tachypnea, skeletal muscle symptoms such as myalgia or rhabdomyolysis, and biochemical abnormalities including liver dysfunction, hypoglycemia, or hyperammonemia [5]. These 2 clinical forms can be sharply separated. Biochemical diagnosis is made by blood acylcarnitine analysis using electrospray ionization tandem mass spectrometry (MS/MS) that measures increases in C4 to C18 acylcarnitine, or through urinary organic acid analysis using gas chromatography mass spectrometry (GC/MS) that detects an increase of ethylmalonic acid, glutaric acid, isovalerylglycine, or dicarboxylic acids. However, it is not always feasible to make a definitive diagnosis of MAD by GC/MS or MS/MS, especially during the stable phase of MAD-M [6]. While genetic diagnosis may represent alternative strategy to make accurate diagnosis of MAD, the genotype/phenotype correlation is not clear.

The usefulness of in vitro probe acylcarnitine assay using cultured fibroblasts and MS/MS for the diagnosis of many FAODs has recently been reported [7]. Other reports showed that severity are associated with acylcarnitine prolife in deficiencies of MCAD, VLCAD, CPT2 and long-chain L-3-hydroxyacyl-CoA dehydrogenase (LCHAD) [8–12]. Subsequent reports also demonstrated that it

☆ This paper was presented at the 34th Annual Meeting of the Japanese Society for Biomedical Mass Spectrometry, Osaka, Japan, 10–11 September 2009.

* Corresponding author. Tel.: +81 853 20 2219; fax: +81 853 20 2215.
E-mail address: yukirin@med.shimane-u.ac.jp (Y. Hasegawa).

1570-0232/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.jchromb.2010.03.029